MECHANISMS OF EPIDERMAL GROWTH FACTOR RECEPTOR SIGNALLING IN PRIMARY RAT HEPATOCYTES

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To my beloved Mum and Dad

献给我最爱的父母

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Abstract

In the U.K. deaths due to liver diseases, especially alcohol related diseases, have risen considerably over the last 20 years. In 2005 up to 13,000 people died from liver related diseases within the U.K., including alcohol and viral liver failure and liver cancers. Worldwide hepatitis B affects about 2 billion people, killing 500,000 to 1 million per year. An effective way to treat liver disease is often liver surgery, such as liver resection for cancers and liver transplant for failure. However, the failure of liver regeneration by hepatocyte proliferation after resection surgery leads to a high death rate, and a shortage of liver donors means most people with liver failure die without access to a transplant. Therefore, understanding hepatocyte proliferation is a key to improving survival after resection surgery and providing hepatocytes for cell therapy in place of organ donation. The mechanism of hepatocyte proliferation has been studied both *in vivo* and in culture by many groups. However, only limited proliferation and preservation of function of primary human and rat hepatocytes, not suitable for clinical use, has been achieved on stimulation with growth factors.

This study focuses on the mechanism of epidermal growth factor (EGF) stimulation of rat hepatocyte cell cycle progression and proliferation, including the role of PI3K/Akt/mTOR and MEK/ERK signalling pathways, EGF receptor location after activation of downstream proteins such as protein kinase B (Akt) and extracellular signal-regulated kinases 1/2 (ERK1/2), and their effect on the cell cycle. Included in this study are some comparisons between the stimulation of the EGF receptor (a tyrosine kinase receptor) and the P2Y receptor (a G protein coupled receptor).

The PI3K/Akt/mTOR signalling pathway appears to be necessary for the hepatocyte response to EGF, inducing progression to S phase and DNA synthesis, while the MEK/ERK pathway is important but not necessary. The $P2Y_2$ agonist UTP, which also stimulates these two pathways, does not result in the cell entering S phase. This suggests that the activation of these two signalling pathways is not sufficient for cell cycle progression. Furthermore, infection of cells with adenovirus to express constitutively active Akt increases hepatocytes proliferation and induces cell cycle progression, which generates a window to obtain hepatocytes proliferation in culture.

It has been shown in this thesis that EGF stimulation of ERK phosphorylation continues from endosomes, while the evidence suggests that UTP stimulation is restricted to signalling at the cell surface. Furthermore, endocytic EGF/EGFR alone (without stimulation from the cell surface) is sufficient to induce cell cycle progression. This endosomal signalling with EGF but not UTP may explain the absence of cell cycle progression following UTP.

EGF stimulates the appearance of phospho-EGFR in the nucleus. Furthermore, nuclear EGFR has a different apparent molecule weight than the cytoplasmic receptor; this may be due to nuclear EGFR having fewer and/or different phosphates. *In vivo* work by others has shown that in liver regeneration following partial hepatectomy (PH) EGF and full-length activated-EGFR were showed to be present in proliferating hepatocytes.

This thesis describes the mechanism of growth factor (EGF) stimulation of primary rat hepatocyte proliferation. It shows for the first time that endosomal EGF/EGFR alone is sufficient to stimulate cell cycle progression, and that EGF induces phospho-EGFR in the nucleus in cultured rat hepatocytes. This thesis also provides the possibility to obtain cultured hepatocytes proliferation including over-expression of constitutively active form of Akt and translocation to the nucleus of full-length EGFR in the phosphorylated form. These studies improve our understanding of growth factor (e.g. EGF) stimulation of hepatocyte proliferation *in vitro* and help to move closer to the goal of obtaining sufficient functional hepatocytes in culture for clinical use, and of drugs that will stimulate hepatocyte proliferation following resection surgery.

Publication arising from this thesis

Papers:

1. Luo, Y., Dixon, CJ., Hall, F., White, PJ., Boarder, MR., "A Role for Akt in Epidermal Growth Factor-Stimulated Cell Cycle Progression in Cultured Hepatocytes: Generation of A Hyperproliferation Window After Adenoviral Expression of Constitutively Active Akt." *J Pharmacol Exp Ther*, 2007, 321: 884-891

2. Luo, Y., Dixon, CJ., Hall, JF., Taylor, E., Boarder, MR., "Internalization of Epidermal Growth Factor Receptor Contributes to EGF-Stimulated Cell Cycle Progression in Primary Hepatocytes: Role of Endosomal Signaling and Nuclear Receptors." *Submitted to Mol Pharm*.

Abstracts:

1. Luo, Y., White, PJ., Dixon, CJ., Boarder, MR., (2006). "Akt inhibitor A-443654 shows Akt is necessary for epidermal growth factor stimulation of hepatocyte proliferation." *75th Anniversary Meeting Abstracts*; *British Pharmacological Society*.

2. Luo, Y., Hall, JF., Dixon, CJ., Taylor, E., Boarder, MR., (2008). "Epidermal growth factor receptors in the hepatocyte nucleus." *August 2008-Vol.22 Abstracts of the EPHAR 2008 Congress, Manchester, UK*

3. Luo, Y., Zhong, C., Dixon, CJ., Hall, JF., Boarder, MR., (2008). "Endosomal signalling from internalised epidermal growth factor receptors in hepatocytes." *August 2008-Vol.22 Abstracts of the EPHAR 2008 Congress, Manchester, UK*.

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List of Abbreviations

2MesADP	2-methylthio-ADP
2MesATP	2-methylthio-ATP
488-EGF	Alexa Fluor® 488 streptavidin conjugated EGF
5-$HT1_A$	5-hydroxytryptamine (serotonin) receptor 1A
A431	human epithelial carcinoma cell line
A443654 (A443)	(S)-1-(1H-indol-3-ylmethyl)-2-[5-(3-methyl-1H-indazol-5-yl)-pyridin-3-yloxy]-ethylamine
AA	arachidonic acid
ADP	adenosine diphosphate
AC	adenylyl cyclase
AG1478	4-(3-Chloroanilino)-6,7-dimethoxyquinazoline
AG825	4-hydroxy-3-methoxy-5-(benzothiazolylthiomethyl) benzylidene cyanoacetamide
Akt	protein kinase B
ANOVA	analysis of variance
AP	anaphase promoting complex
AP	activating protein 1
AP2	adaptor protein 2
AP_3A	diadenosine triphosphate
APS	ammonium persulphate
AT1	angiotensin II (AngII) type 1 receptor
ATP	adenosine triphosphate
BAD	Bcl-2/Bcl-XL-associated death promoter
Bcl-2	B-cell CLL/lymphoma 2
Bcl-xL	basal cell lymphoma-extra large
β-ME	β-mercaptoethanol
BSA	bovine serum albumin
BTC	betacellulin
CAD	carbamoyl-phosphate synthetase 2, aspartate transcarbamylase, and dihydroorotase
CAK	CDK activating kinase
cAMP	cyclic adenosine monophosphate
CDC25	cell division cycle 25
CDK (s)	cyclin-dependent kinase (s)
c-fos	v-fos FBJ murine osteosarcoma viral oncogene homolog
CHO	Chinese hamster ovarian cells
Cip/Kip	CDK interacting protein/Kinase inhibitory protein
CKI	cyclin-dependent kinase inhibitor
c-jun	jun oncogene
c-Met	hepatocyte growth factor receptor
c-myb	v-myb myeloblastosis viral oncogene homolog
c-myc	v-myc myelocytomatosis viral oncogene homolog
CR1	cysteine-rich domains 1
CR2	cysteine-rich domains 2
CT	carboxy-terminal regulatory region

DAG	diacylglycerol
dH_2O	distilled water
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
Dn-Akt	dominant negative Akt
Dn-dynamin	dominant negative dynamin
E1A	early region 1A
E2F	E2 transcription Factor
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
eEF2	elongation factor 2
EGF	epidermal growth factor
EGFR (ErbB1)	epidermal growth factor receptor
EGTA	ethyleneglycol-bis(aminoethylether)-tetraacetic acid
eIF2B	eukaryotic translation initiation factor 2B
eIF4E	eukaryotic translation initiation factor 4E
eIF4G	eukaryotic translation initiation factor 4G
ERK 1/2	extracellular signal-regulated kinase 1/2
Eps8	epidermal growth factor receptor pathway substrate 8
Eps15	epidermal growth factor receptor pathway substrate 15
F.C.	final concentration
FCS	fetal calf serum
FGF	filbroblast growth factor
Foxo	forkhead box class o
G0	gap phase 0
G1	gap phase 1
G2	gap phase 2
GβL	G protein β-subunit-like
GEF	guanine-nucleotide-exchange factor
GPCR(s)	G-protein coupled receptor (s)
Grb2	growth factor receptor-bound protein 2
GS	glycogen synthase
GSK-3	glycogen synthase kinase-3
h	hour/hours
HB-EGF	heparin-binding EGF-like growth factor
HEK-293	human Embryonic Kidney 293 cells
Hepes	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HGF	hepatocyte growth factor
IL-6	interleukin-6
ILK	intergrin-linked kinase
INK4	inhibitors of CDK4
iNOS	inducible NO synthase
IP_3	inositol 1,4,5-trisphosphate
ITS	insulin-transferrin-sodium selenite media supplement
JEG-3	human placental choriocarcinoma cell line
JM	juxtamembrane region
JNK(s)	c-jun-NH_2-terminal kinase (s)
L1	ligand-binding domains 1

L2	ligand-binding domains 2
LAP	latency activated peptide
LPA4	lysophosphatidic acid receptor 4
LTB_4	leukotriene B_4
LY294002 (LY)	2-(4-Morpholinyl)-8-phenyl-4 H-1-benzopyran-4-one
M	mitosis
M199	medium 199
MAPKs	mitogen-activated protein kinases
MBq/ml	megabecquerel per millilitre
MEK (s)	mitogen activated extracellular regulated kinase (s)
MEKK	MAP kinase kinase kinase
MNK1	MAP kinase-interacting kinase 1
MPF	mitosis-promoting factor
MTT	3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium
mSIN1	mammalian stress-activated protein kinase interacting protein 1
mTOR	mammalian target of rapamycin
mTORC2	mTOR Complex 2
myr-Akt	constitutively active Akt
NF-kB	nuclear factors-kB
NGF	nerve growth factor
NLS(s)	nuclear localisation signal(s)
NO	nitric oxide
NTRK1	neurotrophic tyrosine kinase receptor type 1
PBS	phosphate buffered saline
PC12	rat adrenal pheochromocytoma
PCNA	proliferating cell nuclear antigen
PD098059	2-amino-3methoxyflavone
PDGF	platelet-derived growth factor
PDGFR	platelet-derived growth factor receptor
PDK1	phosphoinositol-dependent kinase-1
PEA15	phosphoprotein enriched in astrocytes 15
PH	partial hepatectomy
PI	propidium iodide
PI-3,4,5-P_3	phosphatidylinositol-3,4,5-triphosphate
PI3K	phosphoinositide 3-kinase
PI-4,5-P_2	phosphatidylinositol-4,5-bisphosphate
PI-4-P	phosphatidylinositol-4-phosphate
PKA	protein kinase A
PKC	protein kinase C
PKG	protein kinase G
PLC	phospholipase C
PMSF	phenylmethylsulfonylfluoride
pRb	retinoblastoma protein
PTB(s)	phospho-tyrosine binding domain (s)
PTX	pertussis toxin
PVDF	polyvinylidene fluoride
Rictor	rapamycin-insensitive companion of mTOR
RNA	ribonucleic acid

RNAse	ribonuclease
RTK(s)	receptors of tyrosine kinase(s)
s	second
S	DNA synthesis
S6	ribosomal protein S6
S6K	ribosomal protein S6 kinase
SAPK (s)	stress activated protein kinase (s)
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
Ser	serine
SF	serum-free
SH2	src homology 2
SH3	src homology 3
Shc	src homologous and collagen
Sos	son of sevenless
src	tyrosine kinase src
STAT3	signal transducers and activators of transcription 3
TBS	tris buffered saline
TBS-T	tris buffered saline-tween
TCA	trichloracetic acid
TEMED	tetramethylethylenediamine
TGF-α	transforming growth factor-α
TGF-β	transforming growth factor- β
Thr	threonine
TNF-α	tumour necrosis factor-α
TrkA	neurotrophic tyrosine kinase receptor type 1
Triton	0.1% TritonX-100 in 2% BSA in sterile PBS
TSC2	tuberous sclerosis complex2
Tyr	tyrosine
UDP	uridine 5-diphosphate
UDPβS	uridine 5-O-2- thio-diphosphate
UK	the United Kingdom
UO126	1, 4-diamino-2, 3-dicyano-1, 4-bis (2-aminophenylthio) butadiene
U.S.A.	United States of America
UTP	uridine dinucleotides
WME	Williams Medium E

Chapter 1

Introduction

1.1 Liver

1.1.1 Anatomy

The weight of an adult human liver is normally around 1.4 to 1.6 kilogram. It is a soft, pinkish-brown organ located on the right side of the upper abdomen. It is the second largest organ and the largest gland within the human body.

1.1.1.1. Lobules

The hepatic lobule is a small six-sided cylinder which is considered as the functional unit of the liver. At the periphery, the lobule is surrounded by connective tissue and vessels including the portal vein, hepatic artery and bile duct. At the centre, a branch of the hepatic vein runs through the middle of the lobule. Hepatocytes are arranged as one-cell-thick cords that radiate from the central vein to the edge of the lobule (Figure 1.1).

1.1.1.2 Hepatocytes

The liver consists mainly of hepatocytes which make up 70-80% of the mass of the liver. There are about 2.5 billion hepatocytes in a human liver and approximately every 5000 cells comprise a lobule. The hepatocyte is a polygon with 6-8 sides, each side contains three surfaces: blood-sinusoidal, bile-canalicular and contiguous surface (Figure 1.2). The volume of the hepatocyte changes with changing environment; for example, it increases at low levels of glucose.

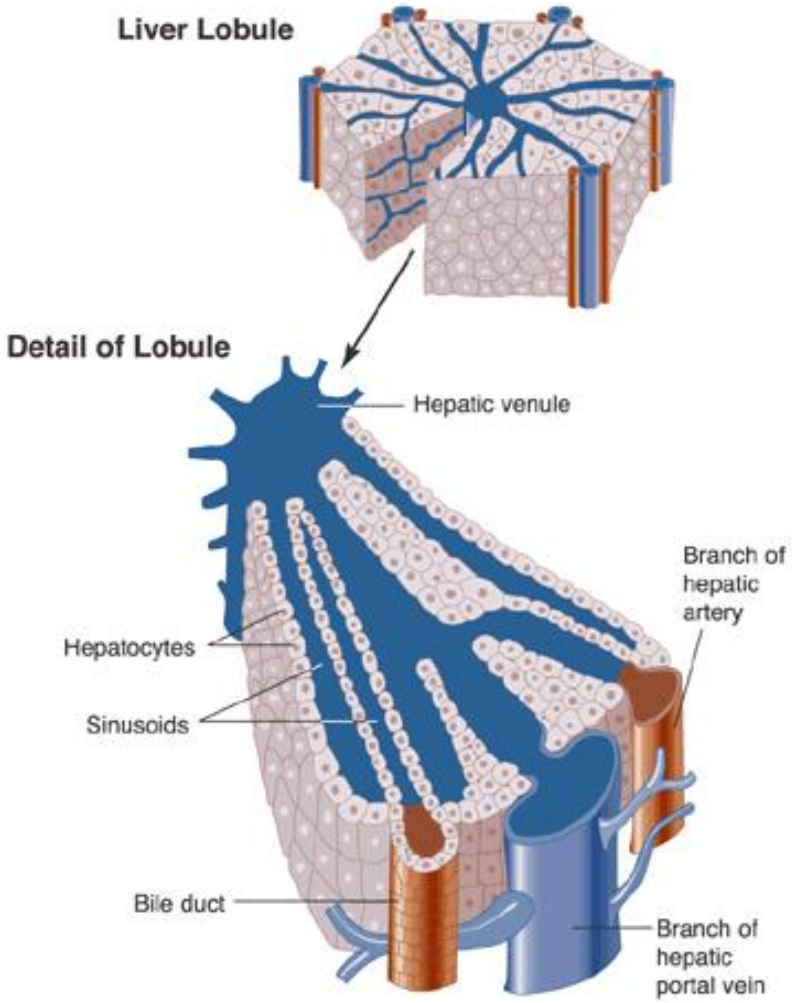


Figure 1.1 Structure of lobules.

(Copy from (Cunningham and Van Horn, 2003)

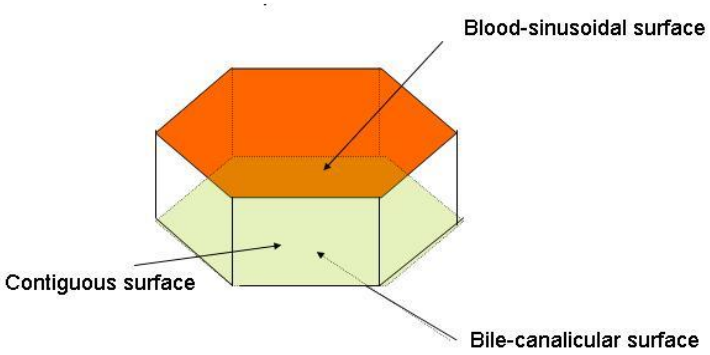


Figure 1.2 Structure of Hepatocyte

1.1.1.3 Sinusoids

The sinusoid is a special small blood vessel which is located in between the cords of hepatocytes (Figure 1.1). The vessel surface consists of endothelicoytes with large gaps between each cell. This structure provides a high permeability to the sinusoid and facilitates the exchange of substance between hepatocyte and blood. Moreover, the hepatic sinusoid is also the place where Kupffer cells develop as an important protection system for the liver due to their phagocytotic ability.

1.1.1.4 Portal area

There are 3 to 4 portal areas in each lobule situated at the corner of each lobule. It is a complex composed of branches including the hepatic portal vein, hepatic artery, bile duct, lymph-vessel and nerves (Figure 1.1).

1.1.1.5 Bile ducts

A bile duct is a long tube-like structure that conveys bile from liver. Bile, which is necessary for food digestion, is excreted by the liver and carried toward the hepatic duct which consists of many small bile ducts. The hepatic duct joins the cystic duct, which leads from the gallbladder, to form the common bile duct, which carries bile into the part of intestine called the duodenum (Figure 1.3).

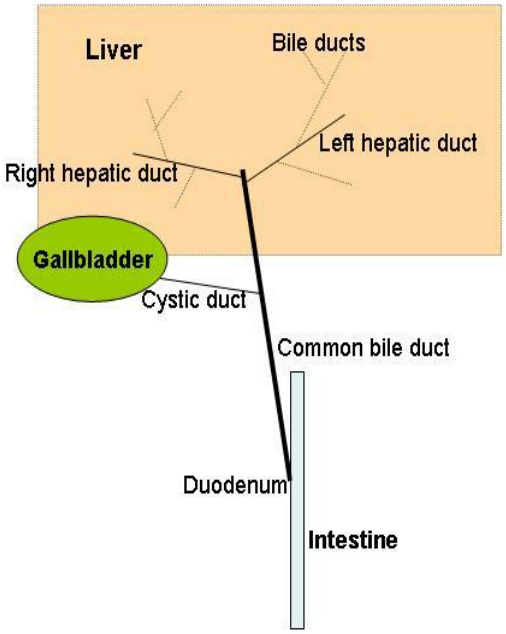


Figure 1.3 Biliary tract

1.1.1.6 Central vein

The central vein is a blood vessel which runs through the middle of each lobule. The blood comes into the liver through the hepatic portal vein and hepatic artery, then drains into the central vein via sinusoids and leaves the liver from the hepatic vein (Figure 1.4).

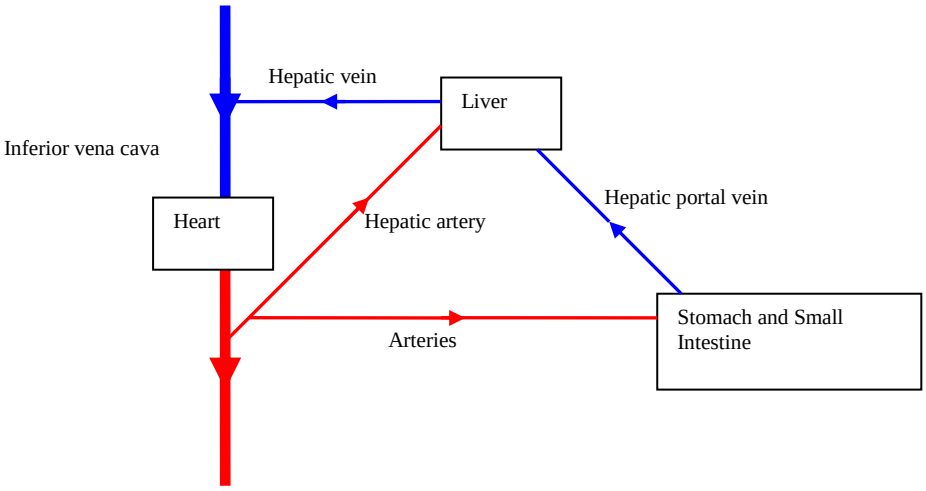


Figure 1.4 Flow of blood between liver and other organs

1.1.2 Physiology

As one of the most important organs, the liver plays a major role in metabolism and has a number of critical functions in the body.

1.1.2.1 Metabolism

The metabolic activity of the liver can be summarized into three major categories: carbohydrate metabolism, fat metabolism and protein metabolism. Carbohydrate metabolism is critical in a human to maintain the proper concentration of glucose in blood. This maintenance of correct blood glucose levels also can be distributed to two mechanisms: short period of time (hours) and long period of time (days to weeks). For instance, in the short term, after a meal, excess glucose in the blood is rapidly converted into glycogen in hepatocytes (a process called glycogenesis); when there is a shortage of glucose in the blood, hepatocytes convert glycogen into glucose and replenish the blood glucose level (a process called glycogenolysis). In the long term, the exhausted

glycogen sources cause hepatocytes to synthesise glucose from amino acids and non-hexose carbohydrates.

Liver is not the unique organ for lipid metabolism in the body but it is largely involved in the fat metabolism. For example, the liver has an ability to produce energy by oxidising triglycerides. The liver also breaks down a large number of fatty acids and produces many acetoacetates for the further metabolism in other tissues. The liver synthesises a large quantity of lipoproteins, cholesterol and phospholipids which are then excreted in bile. Last but not least, the liver is the major organ that converts excess carbohydrates and proteins into fatty acids and triglyceride, which are then stored in adipose tissue.

The most important steps of protein metabolism occur in the liver. Protein is deaminated and transaminated, followed by conversion of the non-nitrogenous part to glucose or lipids by hepatocytes.

1.1.2.2 Glycogen storage

As the second biggest organ in body, the liver is an important site for storage of a multitude of substances including glycogen, vitamin B_{12}, iron and copper. The storage of glycogen, which is critical for the liver regulation of glucose concentration in blood, is one of the most important substances.

1.1.2.3 Plasma protein synthesis

The liver is responsible for the synthesis and secretion of all major plasma proteins (except for antibodies). Albumin, which is important in the regulation of blood volume, is almost only synthesised in hepatocytes. Clotting factors, which are responsible for blood coagulation, are also produced in the liver. Moreover, in the early period of embryo development, red blood cells are mainly synthesised in the liver.

1.1.2.4 Detoxification

The liver is the main site for the removal of toxic substances and most medicinal products from the body. For instance, ammonia, which is very toxic and responsible for central nervous system disease, is converted into urea in hepatocytes.

1.1.3 Diseases of the liver

Liver disease is caused by various agents including virus infection, injury, auto-immunity, hereditary disease, exposure to toxic drugs and alcoholism. For instance, hepatitis, one of the most common liver diseases, is mainly due to infection by the hepatitis A, B and C virus etc. Chronic hepatitis, alcoholism and long-term exposure to toxic drugs always results in cirrhosis, in which the liver generates fibrous tissue to replace dead hepatocytes. In order to treat these diseases, a way to regenerate functional hepatocytes to replace these virus infected cells or fibroblasts needs to be found.

1.1.4 Liver transplantation

Liver transplantation in humans was first performed by Dr Thomas Starzl, who is always called 'the father of modern transplantation', in U.S.A. 1963. Liver transplantation is the only therapy to treat irreversible liver failure such as cirrhosis; also it is a potent way to treat serious liver diseases such as liver cancer. Liver for transplant came from non-living donors until 1989; the first living donor liver transplantation was performed for paediatric liver transplantation. In that operation, 20% of an adult's liver was transplanted into a small child. More recently, adult-to-adult liver transplantation, a strongly controversial operation, has been done. However at least 14 donors died after the surgery indicating the high risk of the therapy (Bramstedt, 2006).

1.2 Liver regeneration

1.2.1 The definition of liver regeneration

Interestingly, the liver is the only human internal organ that has a prominent ability for regeneration. In modern science, human liver regeneration was first recorded over hundred years before (Ponfick, 1890). Since then, many scientists have been involved in the study of the extraordinary regeneration ability of the liver following injury or resection. Although the full mechanism still remains unclear, some scientific advances have been achieved.

In both rodents and humans, the process of 'liver regeneration' after partial hepatectomy (PH) is actually a process of liver compensatory hyperplasia. Instead of

regenerating new hepatic lobules, the remaining lobules of the liver increase their size to restore the total mass of the liver (Bucher and Seaffield, 1964; Michalopoulos and Defrances, 1997; Fausto, 2000; Taub, 2004). During this process, the total mass of the liver is tightly regulated by pro-mitotic and apoptotic mechanisms (Saetren, 1956; Leong *et al*., 1964; Scaife, 1970; Fisher *et al*., 1971). A two-animal combination experiment indicated that the size of the liver may be controlled by the animal weight. In the experiment, parabiotic animals (A and B) have a surgically-generated shared circulation. The removal of 85% of the liver from animal (A) caused compensatory growth of both the remnant (A) and the partner (B) liver (Moolten and Bucher, 1967; Verly *et al*., 1971). Furthermore, when the animal, which 85% of the liver was removed before, was removed from the shared circulation, it resulted in the remainder's (B) liver shrinking to a size required by the animal before which was about 4.5% of the body mass (Moolten and Bucher, 1967). The work also shows the signal for growth come from the circulation. This means it is a hormone or analogue of hormones.

1.2.2 The importance of liver regeneration

Since the liver is an essential organ in the body, the lack of liver regeneration after injury or resection leads to death. In all animal studies including human, failure of the liver to recover its mass invariably causes death. For instance, the impairment of liver regeneration is observed in non-alcoholic fatty liver disease, cirrhosis, chronic hepatic infection, and malnutrition. These diseases increase cell death, delay mitosis and slow the regeneration of normal hepatic mass and in turn result in the failure the regeneration ability of the liver. Therefore, understanding the mechanism of liver regeneration such as the nature of signals and pathways involved is important to treat patients who are

suffering from such liver diseases. In addition, understanding of liver regeneration can increase patient survival rate following PH, which is used extensively in treatment of liver diseases. Last but not least, the advance in the understanding of liver regeneration may lead to new treatments of liver diseases such as liver transplantation.

1.2.3 Review of liver regeneration study

The development of the understanding of the mechanisms of liver regeneration can be simply categorized into three phases. The traditional idea was that a single or several humoral factors played a key role in the response to all of the processes involved in liver regeneration (Fausto, 2000). This was first published in the 1960s by Bucher and colleague who demonstrated that existing 'humoral factors' can induce liver regeneration following PH (Bucher and Seaffield, 1964). In the 1970s, another group identified several humoral factors that played essential roles in liver regeneration including islet cell-derived insulin and some other gut-derived agents (Starzl *et al*., 1973; Starzl *et al*., 1977; Francavilla *et al*., 1978). Also there was the thought that all events required for liver regeneration relied on the activation of one signalling pathway (Michalopoulos and Defrances, 1997). The more recent idea was that liver regeneration was dependent on the activity of multiple signalling pathways (Taub, 2004). The latest model of liver regeneration was much more complicated than any others. Liver regeneration does require the activity of multiple signalling pathways. However, they do not act independently of each other, and there is extensive communication between each signalling pathway. Moreover, the models of interaction between these pathways are particularly complicated since they may occur differently in each liver cell type, some of them may only happen at certain periods of liver

regeneration, and these interactions may involve simultaneous and/or sequential operation (Fausto *et al*., 2006).

1.2.4 Factors involved in liver regeneration

Dependent on their function, the factors involved in the response to liver regeneration can generally be classified into five groups: priming factors, growth factors, co-mitogent, tonic growth inhibitors and suppressors of growth inhibitors.

1.2.4.1 Priming factors

As its name implies, priming factor is the first factor required for liver regeneration following PH. Hepatocytes, which have a very slow turnover (only divide once every year or two), normally cannot respond to hepatic mitogens such as hepatocyte growth factor (HGF) and transforming growth factors-α (TGF-α). Therefore, primary factors are essential to liver regeneration due to their ability to enable hepatocytes to respond to these growth factors. For instance, tumour necrosis factor-α (TNF-α), which is one of the key priming factors, induces transcription of nuclear factors-kB (NF-kB), signal transducer and activator of transcription-3 (STAT-3) in stellate cells and interleukin-6 (IL-6) in Kupffer cells. These activated factors are then translocated to the nucleus, this activates transcription of some immediate early genes, and thereby make hepatocytes competent to respond to various hepatic growth factors (Webber *et al*., 1994a; Webber *et al*., 1998).

1.2.4.2 Growth factors

Growth factors stimulate cells, which have gained replicative competence by priming factors, to progress through the cell cycle from gap phase 1 (G1) to S. The common factors which are involved in liver regeneration include HGF, epidermal growth factor (EGF) and TGF-α.

HGF was first isolated due to its ability to induce hepatocyte deoxyribonucleic acid (DNA) synthesis and was identified as a blood-derived mitogen in culture (Michalopoulos *et al*., 1984; Nakamura *et al*., 1984). HGF activates by binding to its receptor, hepatocyte growth factor recepto (c-Met), and plays the key roles in liver growth and function (Michalopoulos *et al*., 1984). The deletion of either protein's gene expression leads to the incomplete development of liver in mice embryo's and causes death (Schimidt *et al*., 1995). HGF also is important for liver regeneration following PH since its concentration in plasma significantly increases after reduction of the liver mass (Tomiya *et al*., 1992). For instance, in the rat, HGF concentration in plasma rapidly increased to more than 20-fold than normal within 1h after PH and then slowly declined in the following 72h before it was back to normal level (Lindroos *et al*., 1991). Moreover, more recently researches demonstrated that the HGF/c-Met signalling pathway was critical for cell cycle progress after PH by activation of extracellular signal-regulated kinase 1/2 (ERK 1/2) pathway and PI3K/Akt pathway (Burr *et al*., 1998; Pediaditakis *et al*., 2001; Okano *et al*., 2003; Huh *et al*., 2004).

EGF, which is mainly produced in the salivary glands, stimulates the growth of various epidermal and epithelial tissues including liver *in vivo* and *in vitro* (Schlessinger, 2000).

EGF plays an essential role in liver regeneration since inhibiting its secretion by removing salivary glands in rat result in a complete block of liver regeneration after PH (Noguchi *et al*., 1991).

TGF-α, which is a member of EGF family of growth factors, is released by hepatocytes and activated on the cell itself (Mead and Fausto, 1989; Koniaris *et al*., 2003). TGF-α activates the EGFR and induces hepatocyte proliferation. The overexpression of TGF-α gene in mice induced hepatocyte proliferation and led to cancer (Webber *et al*., 1994b). However, TGF-α is not necessary for liver regeneration as inhibition of its gene expression did not effect normal mouse liver regeneration. It indicated that EGF may replace the loss of TGF-α function in the mechanism (Mann *et al*., 1993). On the contrary, TGF-α is suggested as an up-regulation factor to HGF since anti-TGF-α antibodies partially block the regeneration response in the liver (Matsumoto and Nakamura, 1992; Scheving *et al*., 2002).

1.2.4.3 Co-mitogens

Co-mitogens such as insulin promote the activity of the growth factors and allow the progression of the cell cycle.

Insulin is produced by pancreatic islets and is transferred into the liver through the portal vein. There is no direct evidence to show that insulin induces hepatocyte mitosis in normal animals; however the absence of insulin results in the delay of liver regeneration (Starzl *et al*., 1978). Moreover, insulin accelerates liver regeneration after PH (Bucher and Swaffield, 1975; Starzl *et al*., 1977; Hwang and Chen, 1993).

1.2.4.4 Tonic growth inhibitors

Tonic growth inhibitors such as transforming growth factor-β (TGF-β) are known as inhibitors of hepatocyte proliferation (Carr *et al*., 1986). TGF-β is normally secreted from non-parenchymal cells such as sinusoidal endothelial cells and Kupffer cells (Fausto *et al*., 1991). It is down-regulated by priming factors and controls liver regeneration by causing cell apoptosis. The concentration of TGF-β in the plasma increases within 3-4h after PH and then slowly rises until reaching a peak at 48-72h (Braun *et al*., 1988). Since hepatocyte DNA synthesis stops between 48-72h after PH, it suggested that liver regeneration is stopped by TGF-β. Hepatocytes are resistant to TGF-β effects from 12h to 48h after PH (Houck and Michalopoulos, 1989). Also although the injection of TGF-β into the liver after PH caused suppression of peak DNA synthesis at 24h, the whole DNA synthesis response lasts 72h as usual (Russell *et al*., 1988). Recently studies have shown that nitric oxide (NO) may play an important role in the cell resistance to TGF-β (Ronco *et al*., 2004). This ability of hepatocytes to resist TGF-β allows the proliferation of hepatocytes during the time of increasing TGF-β concentration in plasma.

1.2.4.5 Suppressors of growth inhibitors

Suppressors of growth inhibitors inhibit the biological activity of the tonic growth inhibitors. They reversibly bind to the receptors of tonic growth inhibitors with high affinity preventing the tonic growth inhibitors from binding to their cellular receptors. They include TGF-β inhibitor, latency activated peptide (LAP), Activin inhibitor and follistatin.

1.3 Higher eukaryote cell cycle

The cell cycle is the process by which two daughter cells are produced from the parent. In mammals, some cells continuously divide during a life time e.g. bone marrow cells, hair cells, skin cells and blood cells, whereas other cells almost never divide in adults such as neurons and liver cells. The key factors that control cell cycle are cyclins and cyclin-dependent kinases (CDKs). From the present evidence, CDK4 and CDK6 associate with the cyclin D family forming a complex mainly involved in the regulation of the G1 phase. CDK1 associates with cyclin A and cyclin B whose main function relates to the gap phase 2 (G2)/ mitosis (M) phase transition and mitosis. CDK2 is involved in the regulation of G1/S phase transition and DNA synthesis by association with cyclin E and cyclin A respectively (Figure 1.5). There are some other CDKs for which the function is not well understood. For instance, CDK3 may trigger G1/S transition while CDK7 is important for the activation of other CDKs. The cell cycle is also regulated by negative regulation of CDK activity. The major factors involved in the negative regulation of the cell cycle are called cyclin-dependent kinase inhibitors (CKIs). There are two families of CKIs based on their structure and CDK targets. The Cip/Kip (CDK interacting protein/Kinase inhibitory protein) family including $p21^{Cip1/Waf1}$, $p27^{Kip1}$ and $p57^{Kip2}$, which inhibit most cyclin/Cdk complex activation (Figure 1.5); while the inhibitors of CDK4 (INK4) family including $p15^{Ink4b}$, $p16^{Ink4a}$, $p18^{Ink4c}$ and $p19^{Ink4d}$ which inhibit the activation of CDK4 and CDK6 (Figure 1.5) (reviewed in (Pollard *et al*., 2007).

1.3.1 G1 phase

The G1 phase is the gap between the end of M phase and beginning of DNA synthesis phase (S) phase. In most cells, G1 phase is the longest and most variable period of the cell cycle. During G1 phase, various ribonucleic acid (RNA), enzymes and structural proteins, which are required in DNA synthesis and mitosis, are synthesised. For instance, in hepatocytes, growth factors such as EGF trigger the cell cycle at early G1 phase initially inducing the expression of early response genes including v-fos FBJ murine osteosarcoma viral oncogene homolog (c-fos), v-myc myelocytomatosis viral oncogene homolog (c-myc) and ras. These then increase the expression of specific cell cycle regulatory genes which result in the sequential synthesis of D type cyclins (D1, D2 and D3) and cyclin E (Sherr, 1995). At this stage, cyclins are then associated with cyclin-dependent kinases (CDKs) forming active holoenzymes such as cyclin D1/Cdk4 which plays a central role during cell progression through G1 phase. The activated cyclin D1/Cdk4 associates with proliferating cell nuclear antigen (PCNA) in turn stimulating the processing ability of DNA-polymerase-δ (Sherr, 1994). Meanwhile, the cyclin D1/Cdk4 complex inhibits the activation of p21 and p27, which are members of Cip/Kip family that inhibits numerous CDKs (Albrecht *et al*., 1998). Also the cylin D/Cdk4 complex phosphorylates retinoblastoma protein (pRb), a tumor suppressor that is a key repressor of G1 progression that inhibits the expression of cyclin E (Welsh, 2004) (Figure 1.5). The cylin D/Cdk4 induces the phosphorylation of pRb and in turn activates E2 transcription Factor (E2F). The activated E2F results in transcription of various genes such as cyclin E, cyclinA and thymidine kinase etc. Cells may delay progression through G1 phase and enter gap phase 0 (G0) phase when shortage of nutrient or induction of anti-proliferative signal. Finally, progression through G1 phase

is regulated by two checkpoints, the restriction point and the G1 DNA damage checkpoint (Pollard *et al*., 2007). Cyclin E/Cdk2 complex has been reported to play an essential role in driving the cell through restriction point (Pines, 1995; Aleem *et al*., 2005).

1.3.2 S phase

In 1951, DNA replication during one period of the interphase was discovered and named as the synthesis phase (S phase) (Howard and Pelc, 1951). During the S phase, the replication of a chromosome, which is composed of one chromatid, is initiated at many different sites which are referred to as origins of replication. Then the S phase ends with forming two sister chromatids. Also, during DNA replication, actively transcribed regions of the genome replicate in early S phase while inactive regions replicate later. Furthermore, centrosomes and most histones are replicated during S phase (reviewed in (Pollard *et al*., 2007). The S phase is tightly regulated by the association of CDK2 with cyclin E and A. Cyclin E/Cdk2 complex exists during late G1 phase to early S phase and stimulates replication of a pre-assembled initiation complex. After that cyclin E is degraded and CDK2 associates with cyclin A, which activates DNA synthesis from a preassembled replication complex. Furthermore, the cyclin A/Cdk2 complex inhibits the assembly of new replication initiation complexes to ensure that the DNA is only replicated once (Coverley *et al*., 2002) (Figure 1.5).

1.3.3 G2 phase

From the end of S phase until M phase is referred to as G2 phase. During this period, the DNA structure is checked and some RNA and proteins required for M phase such as mircotube proteins are synthesised. There are two checkpoints regulating the progression of G2 phase including the G2 DNA damage checkpoint and the G2 centrosome damage checkpoint (reviewed in (Pollard *et al*., 2007)). The transition of G2/M phase is controlled by the activation of the cyclin A/Cdk2 complex (Malumbres and Barbacid, 2005) (Figure 1.5).

1.3.4 M phase

In 1882, Flemming firstly named the process of nuclear division as mitosis (M phase) which starts after the appearance of the condensed chromosomes, the rest of the cell cycle was referred to as interphase (resting stage). M phase can normally divide into five discretephases. Cell firstly enters prophase, which is characterised by the chromosome condensation and the disappearance of nucleolus. This is controlled by the activation of the cyclin B/Cdk1 complex (Figure 1.5). The protein level of cyclin B increases during G2 to M phase and associates with CDK1 to form the mitosis-promoting factor (MPF). The MPF is activated in M phase by phosphorylation on a threonine residue (Thr161) and dephosphorylation of a tyrosine residue (Tyr15), the activated MPF in turn inititates prophase by stimulating downstream proteins which stimulate chromosome condensation and assembly of the mitotic spindle (King *et al*., 1996). During prophase, chromatin becomes highly organised and condenses to form chromosomes, each of which has two kinetochores, one attached at each sister chromatid, to connect between DNA and microtubes (Chan *et al*., 2005). Also two

centrosomes, which are duplicated during interphase, move to the opposite side of the nucleus. Prometaphase is defined as the formation of the mitotic spindle and the breakdown of the nuclear envelope. It starts with nuclear envelope disassembly and formation of fragments and alveolus in the cytoplasms. The breakdown of the nuclear envelope is also regulated by the cyclin B/Cdk1 complex (Malumbres and Barbacid, 2005). Chromosomes become more highly condensed and both kinetochores on one chromosome are attached to the opposite centrosome by microtubes, forming the mitotic spindle. The chromosome is fully condensed and all chromosomes move to the point midway between two poles in metaphase. Cells then enter anaphase, in which the sister chromatids are separated by the degradation of the proteins that bind sister chromatids together. In this stage, a critical complex involved is the anaphase promoting complex (AP), which is an ubiquitin ligase that promoting degradation of structural proteins associated with the chromosomal kinetochores (Castro *et al*., 2005). The separated sister chromatids then move toward the centrosome to which they are attached, followed by the centrosomes moving apart to opposite ends of the cell. AP also ubiquitinates mitotic cyclins including cyclin A and B and in turn drives cells into telophase (Castro *et al*., 2005) (Figure 1.5). During telophase, the nuclear envelope is reformed around each set of separated sister chromosomes and chromosomes then unfold back into chromatin. Finally, a contractile ring contains actin and myosin develops in the midway between spindle poles and separates the two daughter cells (reviewed in (Pollard *et al*., 2007).

1.3.5 G0 phase

G0 phase is considered as a special compartment of the G1 phase where cell exist in a quiescent state. Cells entry into the G0 is usually due to a lack of growth factor or nutrients and during the G0 phase, cyclins and cyclin-dependent kinases disappear. Cells can re-enter in the cell cycle under certain circumstances such as growth factor stimulation (Pollard *et al*., 2007) (Figure 1.5).

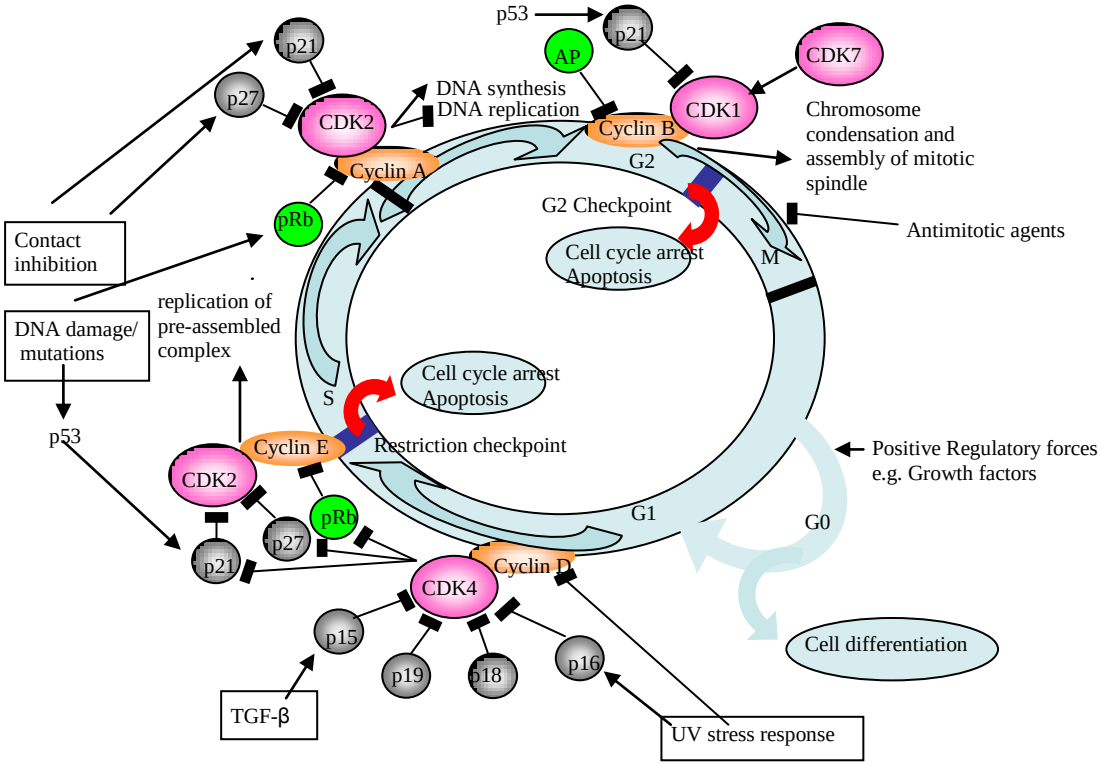


Figure 1.5 cell cycle regulations

(Adapted from Cell Signalling Technology, Schwartz & Shah, 2005 and Rang *et al*, 2007)

1.4 EGF receptor

The EGF receptor family is a member of the large family of receptor tyrosine kinases (RTKs), which activates a wide range of biological responses including mitogenesis, migration, differentiation, apoptosis and dedifferentiation (review of (Wells *et al*., 1998)). In mammals, there are four members of the EGF receptor family: ErbB1 (also called EGFR), ErbB2 (also called c-Neu), ErbB3 and ErbB4. EGFR, the prototype member of this family, is a 170 kiloDalton transmembrane glycoprotein receptor and is widely expressed on many cell types such as epithelial and mesenchymal lineages (Thompson and Gill, 1985). They are involved in multiple biological processes including proliferation, survival, differentiation and metabolism (Schlessinger and Ullrich, 1992). This family is also found to be highly expressed in various cancer cells. For instance, over-expressed ErbB2 causes approximately 30% of invasive breast cancers (Ross and Fletcher, 1998). The over-expression of this family is also found in ovarian and stomach cancers (Salomon *et al*., 1995). The receptor has been reported to be stimulated by at least five genetically distinct ligands including EGF, TGF-α, betacellulin (BTC), epiregulin and heparin-binding EGF-like growth factor (HB-EGF) (Reise and Stern, 1998).

1.4.1 Extracellular domain

As shown in Figure 1.6, the EGF receptor can be divided into three parts: an amino-terminal 622 amino acids extracellular domain, a single alpha-helical transmembrane pass and a 542 amino acids intracellular carboxy-terminal. The extracellular domain consists of four domains which are referred to ligand-binding domains 1 and 2 (L1 and

L2) and two cysteine-rich domains (CR1 and CR2). In them, L1 and L2 are reported to consist of β-helix-folds and form the ligand-binding pocket between them (Garrett *et al*., 1998; Garrett *et al*., 2002; Ogiso *et al*., 2002). Lemmon and his colleagues presented a model in 2003 of which ligand initially binds only L1 in a low affinity state and then leads to a conformational change of the receptor extracellular domain, thereby allowing ligand association with L2 that results in a high affinity binding (Ferguson *et al*., 2003). CR1 and CR2 domains are reported to consist of a number of small modules each of which is held together by one or two disulfide bonds. A conformational change which is due to ligand binding results in the exposure of a loop from the back of CR1, which is proposed to interact with another ligand-bound EGF receptor family member (Garrett *et al*., 2002; Ogiso *et al*., 2002). Additionally CR2 is also considered to play a role in dimer formation and stabilisation (Saxon and Lee, 1999; Berezov *et al*., 2002). Furthermore, it is reported that CR2 is involved in the regulation of binding EGFR to caveolae/raft component of cell membrane which suggests this may regulate the receptor internalisation (Yamabhai and Anderson, 2002). Deletion of CR2 increases the overall activity of EGFR suggesting that it acts as a negative regulatory factor in EGFR activation (Elleman *et al*., 2001).

1.4.2 Transmembrane domain

The EGFR passes the cell membrane through a single alpha-helical domain consisting of 23 amino acids (Figure 1.6). Other than spanning the membrane, additional effects of this small domain have been reported, especially the role of its regulation of receptor dimerisation (Schlessinger, 2002). For instance, it appears to enhance receptor dimerisation after ligand binding in murine B82 cell line (expressing a recombinant

form of EGFR which is missing its cytoplasmic domain) (Tanner and Kyte, 1999). However, mutation in ErbB2 transmembrane domain caused an increase of receptor dimerisation (Bargmann *et al*., 1986). Also exposure of cells to transmembrane domain peptides blocked the receptor auto-phosphorylation and downstream signalling indicating that the transmembrane domain regulating EGFR activity (Bennasroune *et al*., 2004). Furthermore, other groups also have shown that the transmembrane domain regulates the intracellular kinase domain through the rotational twisting dimers (Bell *et al*., 2000; Moriki *et al*., 2001).

1.4.3 Intracellular domain

The intracellular domain comprises three domains: a juxtamembrane region (JM), a tyrosine kinase domain (kinase) and a carboxy-terminal regulatory region (CT) (Figure 1.6). The JM region has been reported to have a number of regulatory functions including regulation of downstream proteins (Castagnino *et al*., 1995) and ligand-dependent receptor internalisation (Kil and Carlin, 2000). There is evidence that the EGFR kinase domain activation loop adopts a conformation similar to the activated insulin receptor kinase domain (Stamos *et al*., 2002). However, distinguishable with other RTKs, EGF receptor family members do not phosphorylate their downstream kinases within kinase domain but in the carboxy-terminal domain. The CT region contains numerous auto-phosphorylation tyrosine residues where there are binding sites to proteins containing src homology 2 (SH2) such as phospholipase Cγ (Nishibe and Carpenter, 1990; Schlessinger, 2000). There are also several tyrosine residues, as well as serine/threonine residues that are phosphorylated by downstream kinases which are part of the EGFR activation cascade (Schlessinger, 2000). For instance, Thr645 is

phosphorylated by protein kinase C (PKC) (Hunter *et al*., 1984), and Tyr845 is a target for tyrosine kinase src (src) (Sato *et al*., 1995). Furthermore, protein kinase A (PKA), a downregulation protein of EGF receptor can phosphorylate several serine residues (Barbier *et al*., 1999). Among these phosphorylated residues, the phospho-tyrosines serve as binding sites for various proteins that contain SH2 or phospho-tyrosine binding domains (PTBs) and recruit proteins to the receptors which in turn regulate various cell signalling cascades (Schlessinger, 2000).

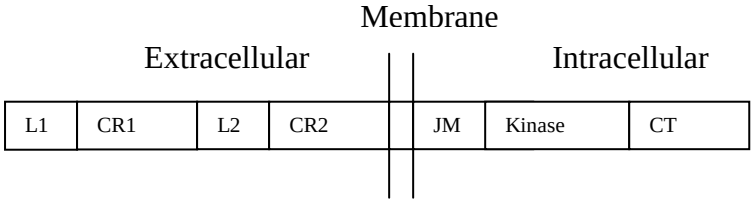


Figure 1.6 Structure of the EGF receptor

1.5 Mitogen-activated protein kinases signalling pathway

Mitogen-activated protein kinases (MAPKs), which are members of a large family of serine/threonine kinases, play a key role in surface receptor regulation of the cell proliferation signalling pathway (Dong *et al*., 2002). Growth factors such as EGF-activated epidermal growth factor receptor (EGFR) recruits the adaptor molecule Growth factor receptor-bound protein 2 (Grb2) directly via a high affinity binding site for molecules with SH2 domains (Madhani, 2001) or indirectly via other components such as src homologous and collagen (Shc) (Plyte *et al*., 2000). The remaining two src homology 3 (SH3) domains of Grb2 regulate the binding of son of sevenless (Sos), a guanine-nucleotide-exchange factor (GEF), which results in GDP/GTP exchange and activates Ras activation (Plyte *et al*., 2000). Activated Ras recruits the cytosolic protein Raf to the membrane and activates it by phosphorylation (Kerkhoff and Rapp, 2001). The activated Raf then phosphorylates specific isoforms of mitogen activated extracellular regulated kinases (MEKs), which in turn activates MAPKs including extracellular signal-regulated kinase (ERK1/2), p38 MAPKs (also termed p38-RK or p38 stress activated protein kinases (SAPKs)) and c-jun-NH_2-terminal kinases (JNK1 and JNK2, also known as p46/p54 SAPKs) (Graves *et al*., 1995; Inglese *et al*., 1995; Seger and Krebs, 1995; Fanger, 1999).

1.5.1 ERK

The ERK1/2, which were first identified in 1990 (Boulton *et al*., 1990), have been well documented and are typically activated by growth-related signals and linked with proliferation and differentiation. Since then, ERK 3, 4, 5, 6, 7 and 8 have been also

identified but are less understood (review in (Bogoyevitch and Court, 2004)). The expression of mutant non-phosphorylatable ERK1/2 abolished CC139 Chinese hamster fibroblasts cell proliferation demonstrating the important role of ERK in cell proliferation (Pages *et al*., 1993). Moreover, inhibition of the activation of ERK by blocking its upstream protein MEK activity by 2-amino-3methoxyflavone (PD098059) completely inhibits rat kidney cell growth (Dudley *et al*., 1995). Not only the activation of ERK but also the translocation into the nucleus is required for growth factor-induced cell cycle progression and gene expression. Pouyssegur and colleague presented the result that the expression of a catalytically inactive form of cytoplasmic MAP kinase phosphoatase was sufficient to sequester ERK in the cytoplasm. Instead of phosphorylation of cytoplasmic substrates, the cytoplasmic ERK inhibited Elk1-dependent gene transcription and growth factor-induced DNA replication (Brunet *et al*., 1999). This observation indicates that ERK translocation into nucleus is required for quiescent cells to response to growth factor and re-enter the cell cycle. Furthermore, ERK nuclear localisation has been demonstrated to be important in the regulation of cell proliferation in the natural environment. For instance, increase of phosphoprotein enriched in astrocytes 15 (PEA15), which regulates export of ERK out of the nucleus, inhibited ERK-dependent transcription regulation and cell (NIH 3T3 cell line) proliferation (Formstecher *et al*., 2001). Also it has been reported that Akt overactivation prevented ERK translocation into the nucleus by stabilising PEA15 and in turn resulted in the inhibition of angiotensin II (AngII) type 1 receptor (AT1) mediated cell (Chinese hamster ovary (CHO) cells) proliferation (Gervais *et al*., 2006).

The activated-ERK is reported to regulate the synthesis of cyclin D1. Inhibition of ERK activity by either expressing a dominant negative MEK (Lavoie *et al*., 1996) or MEK inhibitor PD098059 (Brown *et al*., 1998) was sufficient to block cyclin D1 expression. Furthermore, ERK also regulates cyclin D1 transcription via myc activity (Daksis *et al*., 1994). ERK regulates the formation of the cyclinE/CDK2 complex during G1/S transition. ERK regulates the CDK2 translocating into the nucleus, where it is activated by the phosphorylation of Thr160 by CDK activating kinase (CAK) and dephosphorylation of Thr14 and Tyr16 by the cell division cycle 25 (CDC25) phosphatase (Keenan *et al*., 2001). There is evidence that ERK is also important to cyclinB/CDK1 complex activation. It has been shown that ERK can phosphorylate two of the four phosphorylation sites, all four sites must be phosphorylated for cyclin B1 translocated into the nucleus where cyclinB is activated by phosphorylation on Thr161 and dephosphorylation on Thr14 and Tyr15 (Walsh *et al*., 2003). It appears that ERK also regulates the synthesis of pyrimidine nucleotides in order to synthesise more DNA and rRNA. Evans and colleague reported that ERK increases the activity of the multifunctional enzyme known as carbamoyl-phosphate synthetase 2, aspartate transcarbamylase, and dihydroorotase (CAD), which controls the synthesis of pyrimidine nucleotides (Graves *et al*., 2000). Furthermore, nuclear ERK appears to play an important role since CAD is more phosphorylated in the nucleus than when it is in the cytoplasm. ERK is also shown to regulate protein translation. For instance, MAP kinase-interacting kinase 1 (MNK1), which is a downstream target of ERK, induces the phosphorylation of the translation initiation factor 4E (eIF4E) leading to increased translation (Stefanovsky *et al*., 2006).

1.5.2 c-jun-NH_2-terminal kinase (JNK) and p38 MAPKs

Three genes including *MAPK8, MAPK9 and MAPK10* together form the JNK isoforms. Compared with ERK1/2, JNK appears to be activated by environmental stress and cytokines stimulation and is essential for phosphorylation of activating protein 1 (AP1) (Ip and Davis, 1998). It has been demonstrated that JNK also regulates many normal physiological processes such as cellular proliferation, apoptosis and tissue morphogenesis. For instance, treatment with exisulind (sulindac sulfone) and related compounds induce NIH3T3 cell apoptosis via the protein kinase G (PKG)/ MAP kinase kinase kinase (MEKK)/JNK signalling pathway (Soh *et al*., 2001). Also, p38 MAPK show a crucial role in the regulation of cellular proliferation and differentiation (review in (Fang and Richardson, 2005). However, recent evidence shows that p38 MAPKs may be also important in the regulation of cell apoptosis. It has been reported that the adenoviral early region 1A (E1A)-mediated sensitisation to apoptosis is through inactivation of Akt and activation of p38 MAPKs (Liao and Hung, 2003).

1.6 PI3K/Akt/mTOR signalling pathway

Recent studies demonstrated that the PI3K/Akt/mTOR signalling pathway plays a crucial role in many biological effects including proliferation, differentiation, anti-apoptosis, tumorigenesis and angiogenesis (Review in (Fresno Vara *et al*., 2004). Furthermore, this pathway has been shown to be involved in the regulation of a variety of cancers including breast cancer (Fry, 2001), lung cancer (Lin *et al*., 2001b), melanomas (Krasilnikov *et al*., 1999) and leukaemia (Martinez-Lorenzo *et al*., 2000) etc.

1.6.1 Phosphatidylinositol-3 kinase (PI3K)

PI3K isoforms are divided into three classes: Class I, II and III on the basis of their structure and function (Rameh and Cantley, 1999). Class I PI3K are cytoplasmic heterodimers composed of a catalytic subunit (p110) and an adaptor protein (p85), which contains two SH2 and one SH3 binding domain. The Class I PI3K can be further divided into the Class IA isoforms, which is activated by RTKs, and the Class IB, which is activated by Gβγ subunits of GPCRs. The Class I PI3K are the only ones that are able to convert phosphatidylinositol-4,5-bisphosphate (PI-4,5-P_2) to the second messenger phosphatidylinositol-3,4,5-triphosphate (PI-3,4,5-P_3) (review in (Fresno Vara *et al*., 2004)). Class II PI3Ks, which comprise three catalytic isoforms including C2α, C2β and C2γ, are mainly associated with the phospholipid membranes. They are concentrated in the trans-Golgi network and are present in clathrin-coated vesicles. This class of PI3K produces PI-3,4,5-P_3 from phosphatidylinositol and may also produce PI-3,4-P_2 from phosphatidylinositol-4-phosphate (PI-4-P) (Domin *et al*., 2000). The structure of Class III PI3Ks are similar as Class I, as they are heterodimers composed of a catalytic subunit (Vps34) and a regulatory subunit (p150). They produce PI-3,4,5-P_3 through the conversion of phosphatidylinositol (Volinia *et al*., 1995).

1.6.2 Protein kinase B

Akt is a serine/threonine protein kinase which belongs to the cyclic adenosine monophosphate (cAMP)-dependent protein kinase A/protein kinase G/PKC super family. In mammals, three members of Akt have been identified, Akt1 (Jones *et al*., 1991), Akt2 (Cheng *et al*., 1992) and Akt3 (Brodbeck *et al*., 1999). Akt has been

demonstrated to play a central role in PI3K/Akt/mTOR signalling pathway which is involved in the regulation of growth factor induced cell growth, transcription and cell survival (review in (Brazil and Hemmings, 2001). Akt isoforms consist of three domains: an amino terminal pleckstrin homology domain, a central kinase domain and a carboxyl-terminal regulatory domain.

Akt is activated by Class IA and Class IB PI3Ks, which are activated by RTKs and GPCR respectively (Wymann *et al*., 2003). PI3K produces PI-3,4,5-P_3 which recruits Akt to the plasma membrane to allow subsequent phosphorylation (Andjelkovic *et al*., 1997). A phosphorylation of a conserved threonine residue (Thr308) at the kinase catalytic domain of Akt by the phosphoinositol-dependent kinase-1 (PDK1) is necessary for Akt activation (Stephens *et al*., 1998). Furthermore phosphorylation alone at this residue can partially activate Akt (Alessi *et al*., 1996). An additional phosphorylation at a serine residue (Ser473) in the carborxyl-terminal domain can fully activate Akt. Moreover, the phosphorylation at Ser473 alone has little effect on Akt activation (Alessi *et al*., 1996). However, the mechanism of how Akt phosphorylation at Ser473 is not clear. One theory is that another phosphoinositol-dependent kinase, PDK2 which has been now widely believed is mTOR Complex 2 (mTORC2) (Sarbassov *et al*., 2005) regulates the phosphorylation at Ser473 (Hill *et al*., 2001). Also the intergrin-linked kinase (ILK) was shown to phosphorylate Akt at Ser473 (Delcommenne *et al*., 1998). Dedhar and colleague provided evidence that inhibition of ILK activity by a novel specific inhibitor blocks Ser473 phosphorylation of Akt (Persad *et al*., 2001). Furthermore, recent evidence has shown that tyrosine

phosphorylation may be involved in the regulation of Akt activation (Conus *et al*., 2002; Jiang and Qiu, 2003).

Akt acts as a survival signal by regulation of its downstream targets such as B-cell CLL/lymphoma 2 (Bcl-2)/Basal cell lymphoma-extra large (Bcl-xL)-associated death promoter (BAD) and forkhead box class o (Foxo). Akt induces BAD phosphorylation which is then released from a complex with Bcl-2/Bcl-xL on the mitochondrial membrane. Subsequently, the free BAD associates with 14-3-3 protein in the cytosol and in turn inactivate its pro-apoptotic function (Datta *et al*., 1999). Also, Akt induces Foxo phosphorylation which promotes nuclear export and preventes transcription of growth arrest and pro-apoptotic genes (Skurk *et al*., 2005). Akt also regulates cell metabolism through the activation of glycogen synthase kinase-3 (GSK-3). Akt inhibits the activation of GSK-3 by the phosphorylation of GSK-3 which results in the storage of glucose (Cross *et al*., 1995). Furthermore, Akt may also regulate cell cycle progression and anti-apoptosis via regulation of GSK-3 activity (Review in (Song *et al*., 2005).

1.6.3 Glycogen synthase kinase-3

GSK-3 is a constitutively active serine/threonine kinase which has two isoforms GSK-3α and GSK-3β in mammals. GSK is a downstream target of Akt and can be phosphorylated by Akt on Ser21 of GSK-3α and Ser9 of GSK-3β which results in the inhibition of GSK-3 enzyme activation (Cross *et al*., 1995). GSK is involved in a variety of cell functions including transcription, translocation and cell survival by controlling a number of substrates (review in (Doble and Woodgett, 2003). One of the

most important functions of GSK-3 is the regulation of cell metabolism. GSK-3 inhibits the activity of glycogen synthase (GS). Akt-induced phosphorylation of GSK-3 results in inhibition of GSK-3 activation and in turn activates GS which converts glucose to glycogen. GSK also regulates cell proliferation and survival (Hughes *et al*., 1993). Furthermore, it has been shown that GSK-3 phosphorylates transcription factors such as c-myc, jun oncogene (c-jun) and v-myb myeloblastosis viral oncogene homolog (c-myb) (Plyte *et al*., 1992) and the translation factor eukaryotic translation initiation factor 2B (eIF2B) (Welsh *et al*., 1996). GSK-3 also phosphorylates cyclin D, which stimulates its proteolytic degradation. Therefore, stimulating Akt will inhibit GSK-3 and inhibit cyclin D breakdown, which would contribute to cell cycle progression (Diehl *et al*., 1998).

1.6.4 Mammalian target of rapamycin (mTOR)

mTOR is a serine/threonine kinase that regulates various biological effects including cell proliferation, survival, motility, protein synthesis and transcription (review of (Hay and Sonenberg, 2004)). As the downstream target of Akt, mTOR is activated by Akt-induced phosphorylation of tuberous sclerosis complex2 (TSC2), which in the unphosphorylated form inhibits mTOR activity (Hay and Sonenberg, 2004). However, mTOR involvement in the regulation of Akt phosphorylation on Ser473 also has been reported. mTORC2, which consists of mTOR, rapamycin-insensitive companion of mTOR (Rictor), G protein β-subunit-like (GβL) and mammalian stress-activated protein kinase interacting protein 1 (mSIN1), is probably PDK2 and phosphorylates Akt at Ser473 (Sarbassov *et al*., 2005).

The major function of mTOR is the regulation of translation, especially the recruitment of ribosomes to mRNA. mTOR directly or indirectly regulates the activation of several components of the ribosomes recruitment mechanism including eIF4E which recognises the cap structure at the 5' end of nuclear-transcribed mRNAs; eIF4B and eukaryotic translation initiation factor 4G (eIF4G) which unwind the mRNA 5'-proximal secondary structure to facilitate for the 40S ribosomal subunit binding; ribosomal protein S6 kinase (S6K) and its targets, the ribosomal protein S6 (S6) and elongation factor 2 (eEF2) (review in (Hay and Sonenberg, 2004)). Furthermore, mTOR downstream targets eIF4E and S6K appear to control cell (rat fibroblasts) size and promote cell cycle progression (Fingar *et al*., 2002; Fingar *et al*., 2004).

1.7 Purinergic Receptors

Purinergic receptors were first defined (Burnstock, 1976) and were classified into two major groups: P1 for receptors activated by adenosine and P2 for those activated by nucleotides such as adenosine triphosphate (ATP), as shown in Table 1.1 (Burnstock *et al*., 1978). Based on pharmacology, P2 receptors were divided into two subtypes: P_{2X} and P_{2Y} (Burnstock and Kennedy, 1985). Other P2 receptor subtypes were subsequently named including the P_{2T} receptor which was selective for adenosine diphosphate (ADP) on platelets, the P_{2Z} receptor on macrophages which was activated by ATP (Gordon, 1986) and the P_{2U} receptor which was activated by both uridine dinucleotides (UTP) and ATP (O'Connor *et al*., 1991). In the early 1990s, based on signal transduction mechanisms and cloning studies, two major families were confirmed - the P2X family of ligand-gated ion channel receptors and the P2Y family of metabotropic GPCRs (Abbracchio and Burnstock, 1994). Currently seven subtypes

of P2X and eight subtypes of P2Y receptor (Table 1.2) are recognised (review in (Abbracchio *et al*., 2006).

	P1 receptors	P2 receptors	
Agonist	Adenosine	ATP, UTP, ADP, UDP, UDP-glucose etc	
Subtypes	A_1, A_{2A}, A_{2B}, A_3	$P2X_1$-$P2X_7$	$P2Y_1$-$P2Y_{14}$

Table 1.1 Classification of P1 and P2 receptors

Receptor subtype	Endogenous agonist (human)	Antagonist	Coupling
$P2Y_1$	ADP	MRS 2179	$G_{q/11}$
$P2Y_2$	ATP, UTP	AR-C1 26313	$G_{q/11}$
$P2Y_4$	UTP	Reactive Blue 2	G_i , $G_{q/11}$
$P2Y_6$	UDP	MRS 2578	$G_{q/11}$
$P2Y_{11}$	ATP, NAD^+, $NAADP^-$	Reactive Blue 2, NF 157	$G_{s,}$ $G_{q/11}$
$P2Y_{12}$	ADP	CT 50547	G_i
$P2Y_{13}$	ADP	MRS 2211	G_i
$P2Y_{14}$	UDP glucose, UDP galactose	No antagonists	$G_{q/11}$

Table 1.2 Agonist profile of P2 receptors

(Adapted from Abbracchio *et al*, 2009)

1.7.1 The $P2Y_1$ receptor

The $P2Y_1$ receptor was first cloned and characterised from chick brain (Webb *et al*., 1993) and subsequently from human (Janssens *et al*., 1996), cow (Henderson *et al*., 1995), turkey (Filtz *et al*., 1994), Xenopus (Cheng *et al*., 2003), rat and mouse (Tokuyama *et al*., 1995). The $P2Y_1$ receptor is widely distributed and receptor mRNA is highly expressed in most human tissues including brain, heart, placenta, lungs, liver, skeletal muscle, kidneys, pancreas and various blood cells (Janssens *et al*., 1996; Leon *et al*., 1996; Jin *et al*., 1998). Northern blotting shows a similar result of $P2Y_1$ mRNA distribution in rat (Tokuyama *et al*., 1995; Nakamura and Strittmatter, 1996). In most species, ADP is a more potent agonist than ATP while UTP, uridine 5-diphosphate (UDP) and GTP do not activate the receptor (Waldo and Harden, 2004). When expressed in 1321N1 astrocytoma cells, the rank order of potency is: 2-methylthio-ADP (2MesADP) > ADP = 2-methylthio-ATP (2MesATP) > ATP (Waldo *et al*., 2002). In fact, it has been shown that ATP is a partial agonist at the $P2Y_1$ receptor (Palmer *et al*., 1998) and therefore ATP can act as an antagonist when the receptor expression is at a low level (Leon *et al*., 1997). Recently, by using the purified and reconstituted human $P2Y_1$, it was showen that the receptor couples to $G\alpha_q$ and $G\alpha_{11}$ but not $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$ or $G\alpha_o$ (Waldo and Harden, 2004).

There is a large body of evidence shows that activated-$P2Y_1$ receptor increase the level of intracellular inositol 1,4,5-trisphosphate (IP_3) and releases Ca^{2+} from intracellular stores (reviewed in (Abbracchio *et al*., 2006). Along with $P2X_1$ and $P2Y_{12}$, the $P2Y_1$ receptor plays a major role in platelet physiology (Hollopeter *et al*., 2001). In $P2Y_1$ receptor knockout mice, platelet shape change and aggregation are completely

abolished. Furthermore, the knockout of $P2Y_1$ receptor did not apparently affect the mouse development, survival and reproduction (Fabre *et al*., 1999; Leon *et al*., 1999).

1.7.2 The $P2Y_2$ receptor

The $P2Y_2$ receptor, which previously named as P_{2U} receptor, was first cloned from mouse neuroblastoma cells (Lustig *et al*., 1993). Subsequently, it has been cloned and pharmacologically characterised from human (Parr *et al*., 1994) and rat (Chen *et al*., 1996) cells or tissues. Equivalent concentrations of ATP and UTP fully activate $P2Y_2$ receptors while ADP and UDP are less effective agonists (Lustig *et al*., 1993; Parr *et al*., 1994; Lazarowski *et al*., 1995). It has been shown that UTP induced fast desensitisation of $P2Y_2$ receptors (within 5min) after exposure of cells to agonist. Furthermore, the desensitisation recovered within 5-10min following removal of agonist (Otero *et al*., 2000). Using haemagglutinin A-tagged $P2Y_2$ to track the receptor, it was shown that agonist-induced desensitisation of inositol phosphate responses occurred rapidly following agonist binding to the receptor. Moreover, the receptors were internalised at a slower rate which suggesting that receptor internalisation is not required for agonist-mediated desensitisation (Sromek and Harden, 1998).

The agonist-induced $P2Y_2$ receptor directly couples to phospholipase C (PLC) via $G\alpha_{q/11}$ protein to produce IP_3 and diacylglycerol (DAG) and mediate release of calcium from intracellular stores and PKC activation (reviewed in (Abbracchio *et al*., 2006). $P2Y_2$ receptor activation increases the synthesis and/or release of arachidonic acid (AA), prostaglandins and NO (Lustig *et al*., 1992; Welch *et al*., 2003; Xu *et al*., 2003). The $P2Y_2$ receptor knockout mice demonstrated that the receptors are responsible for

the nucleotide-mediated chloride secretion in airways (Cressman *et al*., 1999; Homolya *et al*., 1999). Furthermore, stimulation of $P2Y_2$ receptor can activate alternative chloride channels and improve mucus clearance in the bronchi (Kellerman *et al*., 2002). Also activated-$P2Y_2$ receptor has been shown to stimulate chloride secretion and increase tear production in the eye (Mundasad *et al*., 2001). There is evidence that $P2Y_2$ induced ERK activation in rat-1 fibroblasts and rat adrenal pheochromocytoma (PC12) cells via a src-dependent transactivation of EGFR (Soltoff, 1998; Soltoff *et al*., 1998). It also has been shown that human $P2Y_2$ receptor may transactivate EGFR or platelet-derived growth factor (PDGF) receptor through two SH3-binding domains in the intracellular carboxyl-terminal tail of the receptor (Liu *et al*., 2004). In addition to induction of ERK activity, the $P2Y_2$ receptor induces the phosphorylation of the stress-activated kinase JNK and p38 (Gendron *et al*., 2003). Activation of $P2Y_2$ causes proliferation of smooth muscle cells (Wilden *et al*., 1998), glioma cells (Tu *et al*., 2000), human lung epithelial tumour cells (Schafer *et al*., 2003) and human epidermal keratinocytes (Greig *et al*., 2003). $P2Y_2$ receptor activation also can induce cell cycle progression in smooth muscle cells from G_1 to S and M phases (Malam-Souley *et al*., 1996; Miyagi *et al*., 1996).

1.7.3 The $P2Y_3$ receptor

The $P2Y_3$ receptor, which is mainly expressed in the brain, spleen and spinal cord, was first cloned and characterised from an embryonic chick brain (Webb *et al*., 1993). It shares 60% sequence homology with $P2Y_6$ receptor and has the same rank order of potency of agonists (Li *et al*., 1998).

1.7.4 The $P2Y_4$ receptor

The $P2Y_4$ receptor was first cloned and characterised from human (Communi *et al*., 1995; Nguyen *et al*., 1995) and subsequently from rat (Bogdanov *et al*., 1998; Webb *et al*., 1998) and mouse (Suarez-Huerta *et al*., 2001). UTP is the most potent agonist at the human $P2Y_4$ receptor (Nicholas *et al*., 1996) while ATP acts as a competitive antagonist (Kennedy *et al*., 2000). However, ATP and UTP equipotently activate the recombinant rat and mouse $P2Y_4$ receptors (Bogdanov *et al*., 1998; Webb *et al*., 1998). In rat, extracellular acidification treatment increases the potency of ATP and UTP at $P2Y_4$ while Zn^{2+} inhibited the ATP induction of $P2Y_4$ receptor activation; furthermore neither treatment affects agonist stimulation of the $P2Y_2$ receptor (Wildman *et al*., 2003). Activation of the $P2Y_4$ receptor stimulate PLC and inhibit adenylyl cyclase, which indicates that the receptor is coupling to $G_{q/11}$ and G_i (Abbracchio *et al*., 2006).

The $P2Y_4$-null mice demonstrated that the knockout of the $P2Y_4$ receptor did not affect the behaviour, growth and reproduction (Robaye *et al*., 2003). The most important function of $P2Y_4$ receptor is mediated via the epithelial response in the intestine (Cressman *et al*., 1999). It has been shown that UTP-mediated epithelial secretion of chloride is mainly regulated by the $P2Y_4$ receptor in mouse colon (Ghanem *et al*., 2005), while both $P2Y_2$ and $P2Y_4$ receptor are involved in the UTP induction secretion of potassium (Matos *et al*., 2005).

1.7.5 The $P2Y_5$ receptor

The orphaned G protein-coupled receptor was originally cloned from activated chicken T cells (Kaplan *et al*., 1993) and subsequently named as $P2Y_5$ receptor (Webb *et al*.,

1996). However, its agonist dATPαS appears not a general ligand for P2Y receptors and further studies showed that the receptor can not be activated by over 40 nucleotides and nucleosides which indicated that $P2Y_5$ may not be a nucleotide receptor (Janssens *et al*., 1997; Li *et al*., 1997).

1.7.6 The $P2Y_6$ receptor

The $P2Y_6$ receptor, which is activated by UDP, was cloned and characterised from mouse (Lazarowski *et al*., 2001), rat (Chang *et al*., 1995) and human (Communi *et al*., 1996). In addition to UDP, uridine 5-O-2- thio-diphosphate (UDPβS) and the uracil dinucleotide are also potent $P2Y_6$ agonists. Furthermore these two agonists do not active $P2Y_1$, $P2Y_2$ or $P2Y_4$ receptors, which show 38%, 44% and 40% sequence identity to the P2Y6 receptor (reviewed in (Abbracchio *et al*., 2006). The $P2Y_6$ receptor-induced IP_3 response is insensitive to pertussis toxin (PTX) inhibition which suggests that the receptor is coupled to $G_{q/11}$ (Chang *et al*., 1995). Unlike the $P2Y_2$ receptor, ligand induced $P2Y_6$ receptor desensitisation and internalisation is slower (30min following exposure to agonist) and the response does not recover following agonist removal. Furthermore, it has been demonstrated that neither PKC nor other Ca^{2+} activated kinases are involved in agonist-induced the $P2Y_6$ receptor desensitisation (Robaye *et al*., 1997; Brinson and Harden, 2001). It has been shown that the P2Y6 receptor widely express in many tissues and/or cells including human splenn, thymus, placenta, intestine and blood leukocytes (Communi *et al*., 1996) and in rat lung, spleen, stomach, intestine and aorta (Chang *et al*., 1995).

$P2Y_6$ receptor message is present in rat smooth muscle cells for which UDP acts as a growth factor (Hou *et al*., 2002). This finding suggests that the $P2Y_6$ receptor may be involved in the regulation of cell proliferation. The $P2Y_6$ receptor regulates contractile effect since UDP and UDPβS inducing the contraction of rat (Malmsjo *et al*., 2003b) and human (Malmsjo *et al*., 2003a) cerebral arteries. Also activation of $P2Y_6$ is involved in the UTP-mediated chloride secretion in rat colonic epithelial cells (Kottgen *et al*., 2003) and human nasal epithelial cells (Kim *et al*., 2004).

1.7.7 The $P2Y_7$ receptor

The $P2Y_{7,}$ which has been shown to be a leukotriene B_4 (LTB_4) receptor (Yokomizo *et al*., 1997), was first identified by low-stringency screening of a human erthroleukemia cell line cDNA library using a $P2Y_3$ receptor encoding probe (Akbar *et al*., 1996).

1.7.8 The $P2Y_8$ receptor

The $P2Y_8$ receptor was cloned from frog embryo (Xenopus laevis) (Bogdanov *et al*., 1997) and may represent an early evolutionary ancestor of $P2Y_2$ and $P2Y_4$ receptors. However, it is not a mammalian receptor.

1.7.9 The $P2Y_9$ receptor

Although the $P2Y_9$ receptor cloning was by homology to other P2Y receptor genes, it has been recently identified as the fourth lysophosphatidic acid receptor (LPA4) (Noguchi *et al*., 2003).

1.7.10 The $P2Y_{10}$ receptor

There is no data which can prove that the $P2Y_{10}$ sequence is a true nucleotide-activated receptor (Ralevic and Burnstock, 1998). The $P2Y_{10}$ receptors only express in lymphoid tissue and it may play a role in B cell development (Rao *et al*., 1997).

1.7.11 The $P2Y_{11}$ receptor

The $P2Y_{11}$ receptor was first cloned and characterised from human placenta cDNA and genomic DNA libraries (Communi *et al*., 1997). The human $P2Y_{11}$ receptor has a unique profile which it is dually coupled to PLC, leading to the release of DAG, IP_3 and intrcellular Ca^{2+}, and also to adenylyl cyclase (AC) to increase cAMP production (Communi *et al*., 1997). The $P2Y_{11}$ receptor is activated by adenine nucleotides such as ATP, which is its natural agonist. However, the potency of ATP activating $P2Y_{11}$ is relatively low since the EC50 is in the 5 to 100μM range whereas in the same expression systems (1321N1 or CHO cells), the EC50 of ATP activation of other P2Y subtypes is in the 10 to 500nM range (Abbracchio *et al*., 2006). The rank order of potency is ARC67085MX ≥ ATPγS ≈ BzATP > dATP > ATP > ADP (Communi *et al*., 1997; Qi *et al*., 2001). There is no evidence that functional $P2Y_{11}$ is expressed in rat or mouse.

1.7.12 The $P2Y_{12}$ receptor

The human (Hollopeter *et al*., 2001; Savi *et al*., 2001), rat (Hollopeter *et al*., 2001) and mouse (Foster *et al*., 2001) $P2Y_{12}$ receptor have been identified and characterised. ADP is the natural agonist of the $P2Y_{12}$ receptor and the rank order of agonist potency is 2MesADP >> ADP > ADPβS in all cases (Abbracchio *et al*., 2006). ATP and its

triphosphate analogs appear to be agonists of the $P2Y_{12}$ receptor in either native $P2Y_{12}$ expressing cells (Simon *et al*., 2001) and heterologously transfected cells (Zhang *et al*., 2001); however, they are antagonists of the $P2Y_{12}$ receptor in platelets (Kauffenstein *et al*., 2004). The $P2Y_{12}$ receptor is mostly expressed in platelets (Hollopeter *et al*., 2001) and also in brain capillary endothelial cells (Simon *et al*., 2001), glial cells (Fumagalli *et al*., 2003), smooth muscle cells (Wihlborg *et al*., 2004) and chromaffin cells (Ennion *et al*., 2004). The $P2Y_{12}$ receptor knockout mice demonstrated that it is important to arrest bleeding and ADP-induced platelet aggregation (Conley and Delaney, 2003).

1.7.13 The $P2Y_{13}$ receptor

Human $P2Y_{13}$ receptor was first cloned (Communi *et al*., 2001) and later characterised (Zhang *et al*., 2002). Subsequently it has been identified and characterised from mouse (Zhang *et al*., 2002) and rat (Fumagalli *et al*., 2004). ADP and AP_3A are natural agonists of the $P2Y_{13}$ receptor while ATP behaves as a weak partial agonist (Marteau *et al*., 2003). 2MesADP is more potent than ADP at human $P2Y_{13}$ receptor whereas ADP is more potent than 2MesADP on the rat $P2Y_{13}$ receptor (Fumagalli *et al*., 2004). The activation of the $P2Y_{13}$ receptor regulates the inhibition of cAMP formation. The $P2Y_{13}$ receptor induced ERK1/2 phosphorylation, accumulation of IP_3 and all the other responses are inhibited by PTX (Communi *et al*., 2001; Marteau *et al*., 2003). This suggests that the $P2Y_{13}$ receptor is coupled to a Gi/o protein. $P2Y_{13}$ mRNA was mostly expressed in spleen, followed by placenta, liver, bone marrow, lung and brain (Zhang *et al*., 2002). The role of the $P2Y_{13}$ receptor is still unclear, however, $P2Y_{13}$-null mice have been recently generated (A. Ben Addi and B. Robaye, unpublished data) and no phenotype has been characterised (review in (Abbracchio *et al*., 2006).

1.7.14 The $P2Y_{14}$ receptor

The $P2Y_{14}$ receptor, which was previously known as GPR105 or UDP-glucose receptor), has been cloned from human myeloid cells, mouse and rat tissue (Chambers *et al*., 2000). The $P2Y_{14}$ can be activated by UDP-glucose, UDP-galactose, UDP-glucuronic acid and UDP-N-acetylglucosamine whereas uridine or adenine nucleotides are inactivate (Chambers *et al*., 2000). In common with the $P2Y_{12}$ and $P2Y_{13}$, it is coupled to G_i protein to inhibit cAMP production (Moore *et al*., 2003). $P2Y_{14}$ mRNA is widely distributed in the human body including placenta, adipose tissue, stomach, intestine, brain, spleen, lung, heart, bone marrow and thymus (Abbracchio *et al*., 2006). In the rat, $P2Y_{14}$ is expressed in the brain and has been reported to be regulated by immunological challenge (Moore *et al*., 2003). This finding suggests that the receptor may be involved the humoral and nervous system response to infection and inflammation.

1.8 Endocytic signalling

Ligand-mediated receptor internalisation has been considered as a mechanism which terminates the activation of the receptor in the past. In the classical model of transmembrane receptor signalling mechanism, association with extracellular ligand results in the receptor at cell surface activating cascades which lead to the cytoplasm and nucleus. The activated receptor is then internalised and degraded. However, new evidence demonstrates that internalisation and formation of signalling endosomes may be important to the full activation of receptor (review in (McPherson *et al*., 2001a).

1.8.1 Growth factor endocytic signalling

The study in liver parenchyma provided clear evidence that the internalised EGFR may be continuing signal from endosomes. Bergeron and colleagues showed that ligand binding to the receptor induced a rapid translocation of the EGFR from the plasma membrane to early endosomes, where the receptor associated with its downstream targets including Shc, Grb2 and Sos (Di Guglielmo *et al*., 1994). Also, it has been reported that nerve growth factor (NGF) associated with its receptor neurotrophic tyrosine kinase receptor type 1 (NTRK1) in endocytic organells (Grimes *et al*., 1996). Furthermore, recent data showed that ligand-bound activated EGFR stimulate the receptor internalisation via activation of Rab5, which is largely associated with early endosomes (Rink *et al*., 2005). Moreover, the same group showed that treatment of EGF also reduced the rate of endosomes disassociation of Rab5 and association of Rab7, which may be the key process that regulates early-to-late endosomes conversation. All these finding suggest a possibility that growth factor receptors such as EGFR may signal from early endosomes after ligand association. Furthermore, internalisation of growth factor may be necessary for the receptor activation. When the EGFR internalisation was inhibited by expression of Dynamin^{K44A} to prolong residence of activated EGF on the plasma membrane, it reduced the receptor activation of its downstream signalling components such as ERK1/2 and the p85 subunit of PI3K (Vieira *et al*., 1996). Further evidences have been provided by using specific reversible inhibitor of EGFR and platelet-derived growth factor receptor (PDGFR) tyrosine kinases to create internalised inactivated non-phosphorylated ligand bound receptors. Then removal of the inhibitor resulted in the recovery of the activation of the

internalised receptor which was able to stimulate further signals and biological effects from endosomes (Wang *et al*., 2002; Pennock and Wang, 2003; Wang *et al*., 2005). These studies are of great important because they show that signalling from endosomes in the absence of signalling from cell surface is sufficient to form certain responses. Moreover, it has been reported that some downstream signalling molecules of growth factor such as epidermal growth factor receptor pathway substrate 8 (Eps8) preferred to association with the EGFR in endosomes rather than on cell plasma membrane (Burke *et al*., 2001). Although the same group also showed that some other downstream signals such as Grb2 were like to associate with the EGFR on the plasma membrane. This finding suggests that EGFR stimulation downstream signals may be from both cell surface and endosomes depending on the targets.

There is considerable evidence that growth factor receptor internalisation is regulated by the activation of clathrin-coated vesicles - the clathrin-mediated internalisation pathway. In the beginning of this process, the adaptor protein 2 (AP2) recruits clathrin to the plasma membrane to allow the conversion of a flat membrane into a bud of high curvature named as clathrin-coated pits (McPherson *et al*., 2001a). The AP2 also recruits ligand-bound receptor such as EGFR to the clathrin-coated pits (Sorkin and Carpenter, 1993). Furthermore, in mutant AP2 overexpressed Hela cell, the number of clathrin-coated pits was significantly reduced and transferrin induced internalisation (clathrin-dependent internalisation) was abolished (Johannessen *et al*., 2006). However, the treatment of EGF resulted in the formation of new clathrin-coated pits and then induced internalisation of the EGFR along with AP2, clathrin and Grb2. There are many evidences show that the clathrin and AP2 directly or indirectly associate with

other endocytic proteins such as amphiphysin I and II (Ramjaun and McPherson, 1998), epidermal growth factor receptor pathway substrate 15 (Eps15) (Owen *et al*., 2000), dynamin (Sever *et al*., 2000) and synaptojanin (Slepnev and De Camilli, 2000) etc, which together regulates the clathrin-mediated internalisation. Recently, other endocytic pathways which are not dependent on the activation of clathrin have been discovered such as caveolae-mediated endocytosis which may be the best studied clathrin-independent internalisation pathway (review in (Mayor and Pagano, 2007). Caveolae are small flask-shaped pits which formatted in the plasma membrane. They are enriched in caveolin, sphingolipids and cholesterol (Simons and Ikonen, 1997). It has been reported that the EGFR may be internalised via caveolae-mediated pathway when stimulation with high concentration of EGF. Sigismund and colleague showed that the high concentration of EGF induced the EGFR internalisation and ubiquitylation via a cholesterol-sensitive (may be caveolae-mediated) pathway; while low concentration of EGF resulted in the EGFR internalisation via clathrin-dependent pathway and did not lead to the receptor degradation (Sigismund *et al*., 2005). They showed that when the EGFR internalised via caveolae-mediated pathway, the EGFR was interacted with the proteins such as eps15 and epsin, which contains ubiquitin-interacting motif. Therefore, caveolae-mediated internalisation pathway leads to the EGFR degradation.

The internalisation of the growth factor may provide a way to allow the receptors to directly activate their target site (Verhey *et al*., 2001). For instance, signals from axon terminals must travel along the axon to the cell body where it activates further processes. Ye and colleagues showed that NGF-induced neurotrophic tyrosine kinase

receptor type 1 (TrkA) internalises and maintains activity in endosomes as it travel along the axon, a processes necessary for neuronal survival (Ye *et al*., 2003). Furthermore, the internalisation may be depending on the proportion of receptors. For example, the EGFR (ErbB1) rapidly internalised in Human epithelial carcinoma cell line (A431) cells after ligand induction, and continue signalled before degradation. Other member of the EGFR family, for instance ErbB2 is preferred to signal from the plasma membrane for a longer period (Sorkin *et al*., 1991; Wiley, 2003).

1.8.2 GPCR endocytic signalling

It has been shown that inhibition of β_2-adrenergic receptor internalisation reduced GPCR-mediated MAPKs (ERK1/2) activation (Daaka *et al*., 1998). This suggested that the receptor internalisation plays an important role in the GPCR activation. Furthermore, that receptor internalisation is required for GPCR-regulated ERK1/2 activation has been found in several other GPCRs including 5-$HT1_A$, μ and δ opioid receptors (McPherson *et al*., 2001a). It appears that β-arrestin, an adapter protein, along with src together regulate the GPCR internalisation and activation, such as induction of ras activation (Luttrell *et al*., 1999).

1.9 Nuclear localisation of EGFR

The EGFR was first claimed to be detected in the nucleus of regenerating hepatocytes in 1991 (Marti *et al*., 1991). Also Raper and colleague reported that EGF translocated to the nucleus during the pre-S phase of liver regeneration (Raper, 1987). There are considerable evidences showed that the EGFR also located in the inner nuclear

membrane of mice liver cells (Klein *et al*., 2004). Furthermore, many groups showed that the nuclear EGFR appears to be as a full-length receptor and can be in the phosphorylated form in many cells and tissues such as Human placental choriocarcinoma cell line (JEG-3) cells (Cao *et al*., 1995), A431 cells (Lin *et al*., 2001a) and breast carcinomas (Lo *et al*., 2005b). Recently evidences suggested that the nuclear translocation may begin with the EGF/EGFR coupled complexes internalisation and inhibition of the receptor internalisation with mutant dynamin blocked the nuclear import of the EGFR (review in (Lo and Hung, 2006). It appears that association with nuclear transport receptors including importin β1 and importin α may be the key for the EGFR translocation from cell surface into the nucleus (DIttmann *et al*., 2005; Lo *et al*., 2005a). Lo and colleague suggested that the nuclear localisation signals (NLSs) within JM of the EGFR interacts to the nuclear transport receptor such as importin β1, which then regulating the receptor translocation into the nucleus (Lo *et al*., 2005a).

The nuclear ErbBs may act as a transcriptional activator was first suggested by Xie and colleagues (Xie 1994). Furthermore, recently findings showed that the nuclear EGFR associated with signal transducers and activators of transcription 3 (STAT3) and then enhanced gene transcription such as cyclin D and inducible NO synthase (iNOS) (Lo *et al*., 2005a).

1.10 Aims and objectives

The main objectives of this study are as following:

* To study the signalling pathway of how cell surface receptor regulates rat hepatocytes proliferation related responses

* To understand how G protein-coupled receptors ($P2Y_2$) and tyrosine kinase receptors (EGFR) signal through PI3K/Akt and MEK/MAPK pathways which leading to different biological response in rat hepatocytes

* To investigate the role of endocytic signal in the full EGFR activation

* To further study the role of internalised EGFR or nuclear EGFR signal to biological response of rat hepatocytes

Chapter2

Materials and Methods

2.1 Materials

Male Wistar Rats were purchased from Charles River. All the medium was obtained from Invitrogen. FCS (fetal calf serum) was supplied by Biosera, BSA, insulin-transferrin-sodium selenite media supplement (ITS) and collagen type 1 (in solutions from rat rail) were supplied by Sigma. Collagenase A was from Roche. Penicillin and gentamicin was ordered from GIBCO. LY294002, 1, 4-diamino-2, 3-dicyano-1, 4-bis (2-aminophenylthio) butadiene (UO126), nystatin, were obtained from Calbiochem. 4-(3-Chloroanilino)-6,7-dimethoxyquinazoline (AG1478), 4-hydroxy-3-methoxy-5-(benzothiazolylthiomethyl) benzylidene cyanoacetamide (AG825), concanovalin A, and filipin were supplied by Sigma. Gefitinib was purchased from AstraZeneca Pharmaceuticals. (S)-1-(1H-indol-3-ylmethyl)-2-[5-(3-methyl-1H-indazol-5-yl)-pyridin-3-yloxy]-ethylamine (A443654 or A443) was a kind gift of Dr Yan Luo, Abbott, Illinois, U.S.A.. The phospho-Akt (Ser473) antibody, Akt antibody, phospho-GSK-3α/β (Ser21/9) antibody and EGFR antibody were obtained from Cell Signalling Technology via New England Biolabs. Rabbit anti-active MAPK pAB, donkey anti-rabbit lgG (H+L) HRP, Anti-Mouse lgG (H+L), HRP conjugate were purchased from Promega. Purified mouse anti-ERK (Pan ERK) mAb was from BD Transduction LaboratoriesTM. Anti-GSK3α/β was produced by Millipore. Phospho-EGFR (tyr1173) antibody was obtained from Santa Cruz Biotechnology. Cy5-affinipure donkey anti-rabbit IgG (H+L) was purchased from Jackson ImmunoResearch Laboratories via Stratech. Transferrin from human serum, Alexa Fluor® 546 conjugate, epidermal growth factor (EGF), biotinylated, complexed to Alexa Fluor® 488 streptavidin conjugated EGF (488-EGF) and SYTOX® green nucleic acid stain were purchased from Invitrogen. All the nucleotides such as ATP, UTP were provided by Sigma. The

adenovirus constructs were a kind gift from Dr Richard Rippe and cultured by Dr John Hall and PhD student Zhong Cheng. All other chemicals were from Sigma and Fisher.

2.2 Methods

2.2.1 Hepatocyte preparation and culture

2.2.1.1 Buffer preparation

Calcium-free buffer was prepared by the addition of 6.77g of NaCl, 0.42g KCl, 0.197g $MgSO_4$, 0.15g NaH_2PO_4, 015g KH_2PO_4, 2.10g $NaHCO_3$, 2.00g Glucose and 0.076g ethyleneglycol-bis(aminoethylether)-tetraacetic acid (EGTA) to 900ml of deionised water. 1M NaOH or 1M HCl was used to adjust the pH to 7.4. The buffer volume was then adjusted to 1L, and the pH was re-checked and re-adjusted, if necessary. Buffer was stored in an autoclaved bottle at 4˚C for up to 1 month.

Calcium-Hepes buffer was prepared by the addition of 6.77g of NaCl, 0.42g KCl, 0.197g $MgSO_4$, 0.15g NaH_2PO_4, 015g KH_2PO_4, 0.35g $NaHCO_3$, 2.00g glucose, 1.8ml of 1M $CaCl_2$ solution and 4.76g 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) to 900ml of deionised water. The buffer was then perfused with the gas (O_2:CO_2, 95%/5% v/v) for 10-15min. 1M NaOH or 1M HCl was subsequently used to adjust the pH to 7.4 before filling the buffer volume to 1L. The solution was stored in an autoclaved bottle at 4˚C for up to 1 month.

2.2.1.2 Cell preparation

Hepatocytes were isolated from male, fed, 200-300g Wistar strain rats following the technique introduced by Berry and Fried (1969) and developed by Seglen (1976). Firstly, the rat was stunned by a blow to the head and killed by cervical dislocation. Immediately following death, a two stage of perfusion was performed at 37˚C by the injection into the hepatic portal using an 18 gauge Monoject needle (Sherwood

Medical). Oxygen was provided into the perfusate by continuous gassing (O_2:CO_2, 95%/5% v/v) of the buffers which also maintained the optimal pH for enzymatic dispersion by collagenase A (pH 7.4). An initial calcium-free perfusion was performed with 150ml of calcium-free buffer during which the liver was dissected out of the rat and suspended above a beaker in a temperature-controlled water-bath. This calcium-free pre-perfusion aids cell dispersion (Seglen, 1972). During the second stage, the liver was perfused on a recirculating basis for 15min with 100ml of the same buffer with calcium (3.8mM), collagenase A (0.04% w/v) and BSA (0.05% w/v). The cells were teased apart from the connective and vascular tissue of the liver by using a scalpel blade to rupture the capsule and the rounded end of a spatula to gently dissociate them, and then filtered through 100μM nylon mesh. The cells were shaken in a temperature controlled water bath at 37˚C to remove dead cells. Then the cells were washed three times in a calcium-Hepes buffer medium by gentle centrifugation at 50g for 2min to remove non-parenchymal and damaged parenchymal cells, and subcellular debris. A final centrifugation yielded a pellet of intact hepatocytes. Cell viability and concentration were assessed by trypan blue exclusion. The cells were diluted at a density of 2 x 10^5 cells/ml in Williams Medium E (WME) containing BSA (0.05% w/v), penicillin (100 units/ml), gentamicin (50μg/ml), ITS, 10% FCS and then plated into collagen coated plates. The cells were incubated in a humidified 5% CO_2 incubator at 37˚C for 4h before changing the medium to serum-free (SF) WME.

2.2.2 Total [^{3}H]-Inositol (Poly) phosphates accumulation assay

The assay is based on the use of lithium, which is an uncompetitive inhibitor of the enzyme-inositol monophosphatase, to block the recycling of inositol in the phosphoinositide pathway and in turn cause the accumulation of inositol phosphates (Fauroux and Freeman, 1999). The cells were labelled to equilibrium [^{3}H]-inositol, which is incorporated into the membrane phospholipids, and total inositol phosphate accumulation was measured in the presence of lithium.

2.2.2.1 Labelling cells with myo-[^{3}H]-inositol

Hepatocytes were obtained and plated in 24-well plates as described before and incubated 4h in WME supplement with ITS and 10% FCS. Then the cells were incubated in 500µl of serum free WME for 20h. Subsequently cells were labelled with myo-[^{3}H]-inositol (0.037Mbq/ml) in serum free medium 199 (M199) (low inositol) with additional penicillin (100units/ml) and gentamicin (50µg/ml) for 48h.

2.2.2.2 Agonist challenge

The plates were transferred to a 37˚C water-bath for all subsequent steps. To 500µl of medium, 50µl of 10mM of Lithium (11X concentration) was firstly added and left for 15min. Subsequently 50µl of agonists (12X concentration) was added to the medium as indicated in each different experiment. The medium was aspirated and immediately replaced with 500µl ice-cold 1M trichloracetic acid (TCA) to terminate the reaction. Plates were then directly transferred onto wet ice for 30min to ensure complete rupture of the cellular membranes and release of the water-soluble phospholipids.

2.2.2.3 Tri-n-octylamine and 1, 1, 2-trichlorofluoroethane extraction

From each well of the 24-well plate, 400µl of the supernatant was transferred into a 5ml polystyrene tube and 100µl of 10mM ethylenediaminetetraacetic acid (EDTA) was also added into the tube to chelate ions such as Ca^{2+}, Na^{+}, K^{+}, Mg^{2+} from the solution. To the resulting solution, 500µl of a freshly prepared 1:1 v/v tri-n-octylamine and 1, 1, 2-trichlorofluoroethane mixture was added. The solution was mixed by vortex, and then microfuged at 10,000g for 10min. Next, 400µl of the upper aqueous phase was carefully transferred into a 1.5ml microfuge tube and 25µl of 250mM $NaHCO_3$ was finally added to the resulting solution to neutralise the pH. Samples were stored at 20˚C or processed further immediately.

2.2.2.4 Separation of total Inositol Phosphates by using the Dowex Columns

A mixture of 1:1 v/v of Dowex and distilled water (dH_2O) was prepared and 2ml of the mixture was added to each polypropylene chromatography column and allowed to settle. The Dowex was then stripped of chloride ions using 2M ammonium formate and recharged with formate ions by the addition of 1M formic acid. The columns were then washed a further 3 times with 10ml dH_2O and then stored at 4˚C covered with sufficient dH_2O.

The columns were washed once with 10ml dH_2O before adding samples. After each sample was added, another 5ml dH_2O was also added into the column. The eluant was run to waste and a further 10ml dH_2O was applied and steps repeated 3 times until the inositol phosphates had attached to the Dowex by exchanging with the formate ions. Next, 10ml of a low concentration of ammonium formate (60mM) was added to the

column to remove the glycerophosphoinositols. Finally total ^{3}H-inositol phosphate fraction was eluted by the addition of a high concentration ammonium formate (4ml, 2M) and collected into the scintillation vials. Following the addition of 15ml FLO-Scintillant to each vial, the samples were vortexed and counted for 3min for each sample. The columns were regenerated by washing with 10ml 1M formic acid after use followed with 3 washes with 10ml dH_2O, then stored as described before until required.

2.2.3 [^{3}H]-thymidine assay

2.2.3.1 Agonist challenge

[^{3}H]-Thymidine assay is a common technique used to measure DNA synthesis, which can be used as an index of cell cycle progression, in cultured cells (Pace *et al*., 1991).

Hepatocytes were isolated and seeded as described before in the 24-well plates and incubated in WME (500µl) with 10% FCS and ITS for 4h. Then serum was removed by changing the medium to SF WME and the cells were further incubated for 24h to allow cells to enter the quiescent G_0 stage of the cell cycle. (In some experiment adenovirus was added and left for 2 or 16h at the beginning of this 24h SF incubation (adenovirus was then removed by washing the cells with SF WME)). Inhibitors such as LY294002, A443654 and AG1478 etc were dissolved in dimethyl sulfoxide (DMSO) to give 1000X the final concentration (F.C.) and then diluted in WME to give 2X the required concentration and mixed well by vortexing. Next, 250µl of medium was gently removed from each well of the 24-well plates and 250µl of 2X inhibitors were added to achieve the F.C. of inhibitors and given a F.C. of 0.1% DMSO. After pre-incubation with inhibitors, cells were then exposed to agonist such as nucleotides and

growth factors which were diluted in calcium-Hepes and WME respectively. To 500µl medium, 100µl 6X agonist (5µl 100X) was added and left 24h. In some experiments (see Chapter 6) inhibitor and agonist (or only agonist) was removed one or several hours after the stimulation by washing the cells with SF WME three times, leaving a 2min gap between second and third wash to allow the cells releasing the inhibitors. Cells were further incubated to achieve a total of 24h incubation from the time of adding agonist, while sometimes inhibitor and/or agonist was added back to cells as described before after an 8h gap from the third wash and further incubated until 24h from the first stimulation. 20h following the first exposure of cells to the agonist, 100µl of WME containing 6µl/ml (7µl/ml) of methyl-^{3}H-thymidine (activity =37MBq/ml) was added to the 500µl (600µl) medium in each well to give a F.C. of 1µCi/ml and incubated for a further 4h to allow incorporation of the radiolabelled nucleotide into the newly synthesised DNA.

2.2.3.2 Extraction of the radiolabelled DNA

Plates were then transferred onto wet ice and the cells were gently washed twice with 1ml ice-cold balanced salt solution (phosphate buffered saline (PBS): 137mM NaCl, 10mM Phosphate, 2.7mM KCl, pH 7.4) with 5min intervals between to remove residual methyl-^{3}H-thymidine. The cells were then washed twice with 500µl ice-cold 5% TCA to precipitate acid-insoluble proteins and DNA and then twice with 500µl ice-cold 100% ethanol. After aspiration of the ethanol the residue was placed in a hood to allow evaporation before the addition of 250µl of 0.5M NaOH which was used as a solvent for DNA. Then the plates were well shaken for 15min and stored at -20˚C if necessary. To this 250µl solution, 200µl was transferred into scintillation vials with

4ml of Emulsifier Safe scintillation fluid added and each vial was vortexed for around 6s until the phases had mixed. The remaining 50µl of solution was used in the protein assay. The radioactivity was determined in scintillation counter for 3min each vial at least 1h after vortex to allow abeyance of background chemiluminescence.

2.2.4 Western blotting

Western blotting is a standard method to detect proteins by electrophoretic separation and electro-transfer of the proteins onto a stable substrate such as nitrocellulose film or polyvinylidene fluoride (PVDF). Subsequently specific antibodies are used to probe the interesting proteins which are visualised by application of a secondary antibody linked to a chemiluminescent or fluorescent dye (Burnette, 1981).

2.2.4.1 Buffer preparation

Lysis buffer (20mM Tris-HCl, 250mM NaCl, 3mM EDTA, 3mM EGTA, 0.5% TritonX-100, pH 7.6) was prepared by the addition of 2ml of 1M Tris-HCl (pH8.0), 5ml of 5M NaCl, 600µl of 500µM EDTA, 600µl of 500µM EGTA and 500µl of TritonX-100 to 80ml of dH2O. 1M HCl or 1M NaOH was used to adjust the pH of the buffer to 7.6. The buffer volume was then made up to 100ml with dH2O, and the buffer pH was re-checked and re-adjusted, if necessary. The solution was stored in 5ml aliquots at -20˚C until use. Directly before use, the inhibitors including 50µl of 100mM phenylmethylsulfonylfluoride (PMSF), 50µl of 200mM Na_2VO_4, 50µl of 2mg/ml aprotinin, 2.5µl of 10mg/ml leupeptin and 5µl of β-mercaptoethanol (β-ME) were added to 5ml of lysis buffer. The solution was stored on ice until use.

Running buffer was prepared by the dilution of 10X running buffer at ratio 1:10 in dH2O. 10X running buffer (25mM Tris base, 250mM glycine, 0.1% sodium dodecyl sulphate (SDS), pH 8.3) was prepared by the addition of 30.3g of Tris base, 187.7g of glycine and 10g of SDS to 600ml of dH2O. It was dissolved completely by using a magnetic stirrer at approximate 50˚C for about 2h. It was then made up to 1L with dH2O. The pH of solution should be approximately 8.3 and it was checked but not adjusted. Solution was stored at room temperature for up to six months.

2X sample buffer (250mM Tris-HCl, 4% SDS, 20% glycerol, 0.008% bromophenol blue and 4% β-ME) was prepared by the addition of 1g SDS, 5ml glycerol, few grains of bromophenol blue and 1ml of β-ME into 20ml of 250mM Tris-HCl (pH 6.8).

Blotting buffer (39mM glycine, 48mM Tris base, 0.037% SDS, 20% methanol, and pH 8.3) was prepared for by the addition of 8.7g of glycine, 17.4g of Tris base, 1.11g of SDS and 0.6L of methanol to 2.4L of dH2O. The pH of solution should be approximate 8.3 and it was checked but not adjusted. This buffer was freshly prepared for each experiment and stored at 4˚C and used within 48h.

10X Tris buffered saline (10X TBS) buffer (20mM Tris base, 137mM NaCl, pH7.5) was prepared by the addition of 24.2g of Tris base and 80g of NaCl to 600ml of dH2O. HCl (37%) or 5M NaOH was used to adjust the pH to 7.5. The buffer volume was then adjusted to 1L with dH2O and the buffer pH was re-checked and re-adjusted, if necessary. Tris buffered saline-tween (TBS-T) buffer was prepared by the dilution of 10X TBS buffer at ration 1:10 in dH2O and addition of tween 20 to give the F.C. of

0.05% v/v e.g. 1ml of tween 20 in 2L TBS. Solution was stored at room temperature for up to six months.

2.2.4.2 Agonist challenge

Hepatocytes were isolated and seeded onto collagen coated 6-well plates as described above. After 4h incubation, the medium was replaced with 3ml SF WME and the cells were incubated for the further 24h or 48h to induce quiescence. In some experiments, adenovirus was added after changing the medium and left for 2h or 16h before removal by washed the cells with SF WME. Prior to stimulation, 1ml of medium was gently aspirated from each well (3ml before taken) at least one hour before exposure of the cells to agonist. Inhibitors were dissolved in DMSO to achieve a 1000 folder of required concentration and diluted in WME to give 2X F.C. and mixed well by vortex. Next, 1ml of medium was gently removed from each well (2ml) of the 6-well plates and 1ml of 2X inhibitors was added and left for a suitable period (15-30min). The cells were then treated with agonists (11X or 100X) as described in each experiment. In some experiments a further wash was required. The cells were washed twice with 3ml SF WME and incubated for about 2min followed with a third wash (2ml SF WME). In some experiments, an acid wash was used to remove extracellular EGF. The cells were placed on ice and washed with cold SF WME, then cells were incubated on ice with cold 0.5M NaCl/0.2M acetic acid for 5min, subsequently cells were washed twice with cold SF WME, finally the cells were removed from the ice and washed once with SF WME at 37˚C. In all the experiments the stimulation was stopped by rapid removal of the entire medium from the plates and immediately freezing at -80˚C for at least 2h.

2.2.4.3 Preparation of cell lysates

The plates were then transferred onto wet ice and 100µl ice-cold lysis buffer was added to each well. Cells were detached from the plate using a scraper which was washed with dH2O and 100% ethanol between using on each well. The solution was transferred to a 1.5ml microcentrifuge tube and sonicated on ice for 5min and then microfuged at 13000rpm for 10min at 4˚C. The supernatant was transferred to an Eppendorf and vortexed. Appropriate (the remaining volume was enough for the protein assay) volume was transferred to another Eppendorf and an equal volume of 2X sample buffer was added and heated at 105˚C for 5min before storage at -20˚C until assay, while the rest was kept for protein assay.

2.2.4.4 SDS-Polyacrylamide gel electrophoresis

Polyacrylamide gels (0.75mm thick) consisted of a 5% stacking gel (5% 1:30 acrylamide, 0.125M Tris-HCl pH 6.8, 0.1% SDS, polymerised with 0.1% ammonium persulphate (APS) and 0.1% tetramethylethylenediamine (TEMED)) and a 7% or 10% running gel (7% or 10% acrylamide, 0.375M Tris-HCl pH 8.8, 0.1% SDS, 0.1% APS and 0.0625% TEMED).

Protein-equalised samples (re-heated 5min at 105˚C) were loaded to achieve 10-40µg (volume not greater than 25µl) each lane and 5µl Bio-Rad Precision Protein Standards (a range of 250, 150, 100, 75, 50, 37, 25, 15 and 10kDa) was loaded into the first lane and sample buffer into any unused lanes. Samples were firstly run through stacking gel at 150V for 15min using Bio-Rad Protein II mini-gel system to focus proteins into a

narrow band. Subsequently proteins were electrophoretically separated on the running gel at 100V for approximately 1h until the dye front reached the end of the gel.

2.2.4.5 Electro-transfer of proteins

The running gel was cut away from the mini-gel system and transferred onto a pre-soaked membrane PVDF soaked in methanol 5s then 30min in water and cold blotting buffer 15min). Next, the gel and membrane were sandwiched between 6 sheets of blotting paper and 2 fibre pads (soaked in blotting buffer for 15min) and enclosed in the blotting cassette. Wet blotting was performed in a Bio-Rad Trans-blot cell which was filled with cold blotting buffer. The proteins were transferred to membrane (from cathode to anode) at 100V for 1h under cooled conditions.

2.2.4.6 Probing with antibodies

The membrane was washed 5min within TBS-T and transferred to a suitable size container and blocked in blocking agent e.g. 2% BSA or 5% milk (dissolved in TBS-T) for 1-2h at room temperature with a gentle shaking. The membrane was washed with TBS-T six times for 5min each wash on a rocking platform. The blot was then soaked in primary antibody at a required dilution in suitable diluent (5% milk or 0.1% BSA) and gently rocked overnight at 4˚C. The next day, the blot was washed by two times of 1min washes, six times of 15min washes with TBS-T on a well-shake rocker at room temperature to rinse out the excess antibody. After that, secondary antibody (diluted in 5% milk or 0.1% BSA) was incubated with the blot at room temperature for 1h with a gentle rock. The excess secondary antibody was washed out in the same way as performed after primary antibody. The membrane was then drained off to remove

excess TBS-T and immediately soaked in enhanced chemiluminescence (ECL) Plus™ reagent and rocked in dark at room temperature for 5min. Next, the membrane was wrapped in cling film and exposed to the film in an extremity cassette in the dark room for an appropriate time (10s to 1h). Finally, the film was developed and fixed using Kodak GBX developer and replenisher.

	Blocking agent	Primary antibody	Secondary antibody
Phospho-ERK	2% BSA/TBS-T	1:5000 in 0.1% BSA /TBS-T	1:35000 donkey anti-rabbit in 0.1% BSA /TBS-T
Pan-ERK	2% BSA/TBS-T	1:1000 in 0.1% BSA /TBS-T	1:35000 donkey anti-mouse in 0.1% BSA /TBS-T
Phospho-Akt	2% BSA/TBS-T	1:1000 in 0.1% BSA /TBS-T	1:20000 donkey anti-rabbit in 0.1% BSA /TBS-T
Pan-Akt	2% BSA/TBS-T	1:1000 in 0.1% BSA /TBS-T	1:20000 donkey anti-rabbit in 0.1% BSA /TBS-T
Phospho-GSK	5% milk/TBS-T	1:1000 in 5% BSA /TBS-T	1:10000 donkey anti-rabbit in 5% milk/TBS-T
Pan-GSK	5% milk/TBS-T	1:1000 in 5% BSA /TBS-T	1:10000 donkey anti-rabbit in 5% milk/TBS-T
Phospho-EGFR	5% milk/TBS-T	1:1000 in 5% BSA /TBS-T	1:10000 donkey anti-rabbit in 5% milk/TBS-T
Pan-EGFR	5% milk/TBS-T	1:1000 in 5% BSA /TBS-T	1:10000 donkey anti-rabbit in 5% milk/TBS-T

Table 2.1 Concentration, blocking agent and dilution of each antibody

2.2.5 Bradford protein assay using 96-well plate

The Bradford protein assay is used to measure the shift in the absorption maximum from 465nm to 595nm caused by the reaction between Coomassie Brilliant Blue G-250 dye and protein (Bradford, 1976). The standard concentration series was produced by dissolution of suitable volume of 1mg/ml BSA to dH2O to obtain a concentration range from 0 to 1mg/ml at each 0.1mg/ml. Bradford reagent was added to the standard protein and unknown sample and the absorbance was measured at 595nm after mixing the protein and reagent on a shaker for approximately 30s and further incubating at room temperature for about 5min. The unknown sample was diluted to around 0.3mg/ml-0.7mg/ml and then the reading was compared to the standard curve to achieve the concentration.

2.2.6 MTT assay

The technique is based on the tetrazolium ring of 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium (MTT) which is cleaved by mitochondrial dehydrogenases in living cells to generate a purple formazan. The purple formazan is then dissolved in solubilization solution, such as DMSO, and quantified by measuring at 540nm. Since the reduction can only be induced by activated mitochondrial reductase enzymes, the assay can reflect the number of viable cells (Mosmann, 1983).

Hepatocytes were plated on collagen coated 24-well plates (500μl) as described before. If adenovirus was involved, cells were incubated with virus as described in [^{3}H]-thymidine assay. After 24h SF incubation, the cells were treated with inhibitors and

agonists in the same way described in [^{3}H]-thymidine assay. 22h after the cells exposed to agonist, 125µl of MTT (2mg/ml in PBS) was added to each well (500µl) and further incubated for 2h. All medium was aspirated without disturbing the purple solid, and the formazan was solubilised in 350µl DMSO with a 1min shake. Finally, the absorbance was measured at 540nm using a UV microplate reader.

2.2.7 Immunocytochemistry and confocal work

2.2.7.1 Agonist challenge

Hepatocytes were produced and seeded in collagen coated 12-well plates (1ml) with 16mm plastic coverslips as described above. The cells were then incubated in SF WME for 24h to induce quiescence. When the adenovirus was required, the cells were incubated with virus for 2h or 16h of the beginning of the 24h SF incubation and removed by washing the cells with SF WME. Inhibitors were firstly dissolved at 1000X concentration in DMSO and diluted in WME to obtain 2X concentration. To 1ml of medium, 500µl was removed and 500µl of 2X inhibitor solution (or WME) added and cells were further incubated for a suitable time (15-30min). Then 2µl of EGF (200µg/ml) or 488-EGF (200µg/ml) was added to the medium (1ml). In some experiments, EGF was firstly diluted to 11X concentration before adding to medium to achieve 3nM F.C. When a wash was required, it was performed as described in the [^{3}H]-thymidine assay.

2.2.7.2 Immunocytochemistry staining

The cells (on coverslips) were quickly washed with cold PBS before fixing in freshly prepared cold 3.7% paraformaldehyde in PBS for 15min at 4˚C. This was followed with a rapid wash in cold PBS. The cells were then permeabilised in Triton (0.1% TritonX-100 in 2% BSA in sterile PBS) at room temperature for 2min. After that the cells were washed three times with cold PBS (two quick washes with 5min between each wash) on ice to remove Triton from the cells. 100µl of blocking buffer (10% donkey serum in 2% BSA/PBS) was pipetted onto each converslip and incubated for 1h at room temperature. The blocking buffer was removed and replaced with 80µl primary antibody (1:100 anti-EGFR or 1:50 anti-P-EGFR in 2% BSA/PBS). Following incubation overnight at 4˚C, primary antibody was washed as performed after permeabilisation. The secondary antibody (Cy5-donkey anti-rabbit, 1:1 diluted in Glycerol and then 1:200 in 2% BSA/PBS) was then applied to the cells. And secondary antibody off, washes were applied to the cells as described after permeabilisation. In some experiments, a nuclear stain was applied such as propidium iodide (PI) (50µg/ml in PBS), which was incubated with the cells for 30min at 37˚C with ribonuclease (RNAse) (10µg/ml) supplement, or SYTOX green (100nM in PBS), which was incubated with the cells for 30s at room temperature (Suzuki *et al*., 1997; Monga *et al*., 2002).

2.2.7.3 Mounting and observation

The coverslips were fixed on microscope slides around the edge by using nail polish, while Citi-fluor was added to protect the fluorescent signal. In the experiment that did not require the application of antibody, coverslips were directly mounted after fixing.

The slides were then observed under confocal microscope (Leica) with the same setting of offset and gain applied to every slide from one experiment.

2.2.8 Preparation of adenovirus lysis solution

Human Embryonic Kidney 293 (HEK-293) cells were grown in T175 cm^2 flasks until 90% confluent. Then the HEK-293 cells were infected with adenovirus encoded encoding dominant negative Akt (Dn-Akt), constitutively active Akt (myr-Akt) or dominant negative dynamin (Dn-dynamin) at 1:1000 dilutions. Subsequently incubation of the cells with adenovirus at 37˚C (O_2:CO_2, 95%/5% v/v) until cytopathic effect was detected, typically 3-5 days. The cells were then harvested by shaking to gently loosen and transferred to centrifuge pots, spin at 2000rpm for 10min. Supernatant were removed, followed washing remains with PBS and then resuspend the cell pellet in 1ml of PBS. Cells were lysed by three cycle of freezing/thawing (liquid nitrogen/37˚C water bath) treatment. Finally, cellular debris was removed by centrifugation at 2500rpm for 10min and the supernatant was aliquoted and stored at -80˚C until used.

2.2.9 Data analysis

Image J (National Institutes of Health, U.S.A.), a Java-based image processing program, was performed to quantify the level of signal from western blotting image. Data analysis was performed using GraphPad Prism (GraphPad Software Inc, San Diego, CA). Data was presented in figure and text as mean ± standard error of the mean (SEM). For comparison, one-way analysis of variance (one-way analysis of variance

(ANOVA)) or two-way analysis of variance (two-way ANOVA) was used for multiple comparisons. In the case of significantly difference, further analysis was performed including Dunnet's (compares all data sets to the control) and Bonferroni's post-test (compares selected data sets). In all cases, data was considered as significant different if P values were less than 0.05.

Chapter 3

The Effect of Growth Factor on Primary Rat Hepatocytes

3.1 Introduction

Growth factors such as EGF and HGF are considered to be the primary inducers involved in the stimulation of hepatocyte proliferation and liver recovery *in vivo* (Matsumoto and Nakamura, 1992; Huh *et al*., 2004; Michalopoulos and Khan, 2005; Fausto *et al*., 2006). In the early 1980s, it has been reported that PH down-regulated the phosphorylation of EGF and EGFR activity (Rubin, 1982). Furthermore, low levels of EGF in the plasma following removal of the salivary glands in male mice resulted in the failure of liver regeneration after PH (Jones *et al*., 1995). The essential role of HGF and c-Met during liver regeneration was demonstrated by inducing mutated c-Met expression in adult mice (Borowiak *et al*., 2004). They showed that in the Mx-cre transgene induced c-Met mutant mice the liver regeneration response after PH was significantly reduced. Limited proliferation and retention of function of primary human or rat hepatocytes in culture has been achieved on stimulation with growth factors (Loyer *et al*., 1996; Runge *et al*., 1999; Bell *et al*., 2000; Runge *et al*., 2000a; Runge *et al*., 2000b; Paine and Andreakos, 2004; Serandour *et al*., 2005; Yamasaki *et al*., 2006). For instance, EGF induced hepatocyte cell cycle progression (Loyer *et al*., 1996); moreover it have been reported that a small number of cells divided during culture after EGF treatment (Yamasaki *et al*., 2006). Also, it has been demonstrated that primary hepatocytes could retain their functions including the storage of glycogen, expression of plasma proteins and response to growth factor stimulation up to at least 36 days in culture (Runge *et al*., 1999; Runge *et al*., 2000a; Runge *et al*., 2000b).

In the work described in this chapter two growth factors, EGF and HGF, both of which have been reported to stimulate hepatocyte proliferation *in vivo* and *in vitro*

(Michalopoulos and Khan, 2005; Fausto *et al*., 2006), were compared for their ability to stimulate primary rat hepatocytes *in vitro*. ITS (Insulin-transferrin-sodium selenite media supplement from Sigma), which is used as a supplement during cell culture, has been shown to improve the survival of isolated cells (Hermann *et al*., 2000; Lawlor and Alessi, 2001), and therefore the effect of ITS on hepatocytes was also investigated. EGF was further studied to investigate in more detail of the possible mechanisms involved in its stimulation of hepatocytes. These studies involved determination of the time course of EGF induction of DNA synthesis, cell proliferation and the phosphorylation of Akt and ERK, since Akt and ERK were demonstrated to play essential roles in the stimulation of hepatocyte proliferation and liver regeneration by growth factor (Roberts *et al*., 2000; Coutant *et al*., 2002). For instance, it has been shown that the inhibition of ERK activity, by which blocking the up-regulation of MEK with PD098059, reduced the ability of the EGF to suppress TGF-β1 induced apoptosis in primary hepatocytes (Roberts *et al*., 2000). This indicated a key role of ERK in EGF-mediated survival signalling pathway. Also when Akt activity was blocked by inhibition of PI3K using LY294002, EGF did not significantly reduce the TGF-β1 induced apoptosis of rat hepatocytes. Furthermore, when Akt activity was inhibited, hepatocytes were more sensitive to TGF-β1 induced apoptosis (Roberts *et al*., 2000). This suggested that PI3K/Akt pathway played a critical role in both normal and EGF-mediated protection of hepatocytes to apoptosis. However, Baffet and his group presented a slightly different result. They claimed that the PI3K/mTOR pathway is only essential for hepatocyte proliferation while the MEK/ERK response is crucial to both proliferation and survival (Coutant *et al*., 2002). They showed that blocking of the activity of either PI3K with LY294002 or MEK with U0126 completely inhibited EGF

induction of DNA synthesis in primary rat hepatocytes. Furthermore, the inhibition of MEK/ERK signalling pathway reduced the ability of EGF to protect hepatocytes from apoptosis whilst the inhibition of PI3K activity had no effect (Coutant *et al*., 2002). However, both groups provided strong evidences that the PI3K/Akt and MEK/ERK pathways are important for hepatocytes in response to EGF stimulation of proliferation- and/or survival-related effect. Therefore the role of PI3K/Akt and MEK/ERK signalling pathways was studied using a number of highly specific inhibitors and mutant proteins. The activity of PI3K was inhibited by LY294002, a highly selective inhibitor of PI3K (Vlahos *et al*., 1994; Semba *et al*., 2002), and the effect of EGF on its downstream protein-Akt and cell cycle progression were investigated. The role of mTOR on EGF stimulation of cell cycle progression was also investigated by using rapamycin, a selective inhibitor of mTOR (Gingras *et al*., 2001), to block the activity of the mTOR and DNA synthesis was measured by the [^{3}H]-thymidine assay. UO126, which is a dual MEK1 and MEK 2 inhibitor (Favata *et al*., 1998), was supplied and the effect of EGF on MEK down-regulated protein-ERK and hepatocytes DNA synthesis was measured. In order to study the effect of Akt on the EGF stimulation in hepatocytes, A443654 which is a novel inhibitor of Akt (Luo *et al*., 2005), was used to inhibit Akt activity, then the phosphorylation of GSK-3 which is a downstream protein of Akt and DNA synthesis were investigated by western blotting and [^{3}H]-thymidine assay respectively. Furthermore, cells were transfected by adenovirus to express Dn-Akt and myr-Akt and the level of [^{3}H]-thymidine incorporation and cell viability was measured.

Since the EGFR is member of the tyrosine kinase superfamily of receptors (Thompson and Gill, 1985; Wells, 1999), the effect of tyrosine kinase inhibition was also investigated. It was reported that ligand-binding causes both homo-dimerisation and hetero-dimerisation of EGFR (also known as $ErbB_1$) with itself and other family members such as $ErbB_2$, which has been reported as a major binding partner for EGFR (Tzahar *et al*., 1996; Graus-Porta *et al*., 1997), followed with auto-phosphorylation of the kinase domain of the receptors. Furthermore, it have been reported that the complex was more stable at the cell surface when it contained $ErbB_2$ (Lenferink *et al*., 1998). Also, Yarden and his colleagues claimed that $ErbB_2$/EGFR were preferentially recycled and re-activated on the cell surface after internalisation whereas EGFR homo-dimers were usually undergone degradation (Levkowitz 1998). Therefore $ErbB_2$/$ErbB_1$ signalling was considered as more extensive and potent than EGFR homo-dimers-regulated signalling. In this chapter, two tyrosine kinase inhibitors were used, AG1478 which inhibits EGFR homo-dimerisation dependent tyrosine kinase activity and AG825 which has been reported to inhibit (EGFR/$ErbB_2$) tyrosine kinase activities (Osherov *et al*., 1993; Osherov and Levitzki, 1994). The effect of the AG1478 or AG825 inhibition on the EGF induction of the phosphorylation of EGFR and cell cycle progression were measured to investigate which dimer ($ErbB_1$/$ErbB_1$ or $ErbB_1$/$ErbB_2$) is responsible for EGF stimulation in primary rat hepatocyte.

3.2 Results

3.2.1 Comparison of EGF and HGF concentration-response curve effect on the phosphorylation of Akt and ERK

In Figure 3.1, cells were exposed to different concentrations of EGF or HGF for 20min and Akt and ERK phosphorylations was measured using western blotting. To study only the growth factor effect on the stimulation of the phosphorylation of Akt and ERK, cells were not treated with ITS as usually done in other experiments. The results showed that EGF was more potent than HGF at stimulating phosphorylation of Akt and ERK. EGF stimulation of the phosphorylation of ERK was evident at 300pM and reached a peak at 3nM, while the HGF induction of ERK phosphorylation was firstly detected at 1nM. Furthermore 3nM EGF also resulted in an increase of the level of Akt phosphorylation, whereas 3nM HGF did not clearly stimulate the phosphorylation of Akt in primary rat hepatocytes. Therefore, EGF was further studied due to its greater stimulation of ERK and Akt phosphorylation. A concentration of 3nM EGF was chosen as it gave an optimal stimulation of phospho-Akt and phospho-ERK and was comparable to accepted physiological concentrations (Jones *et al*., 1995).

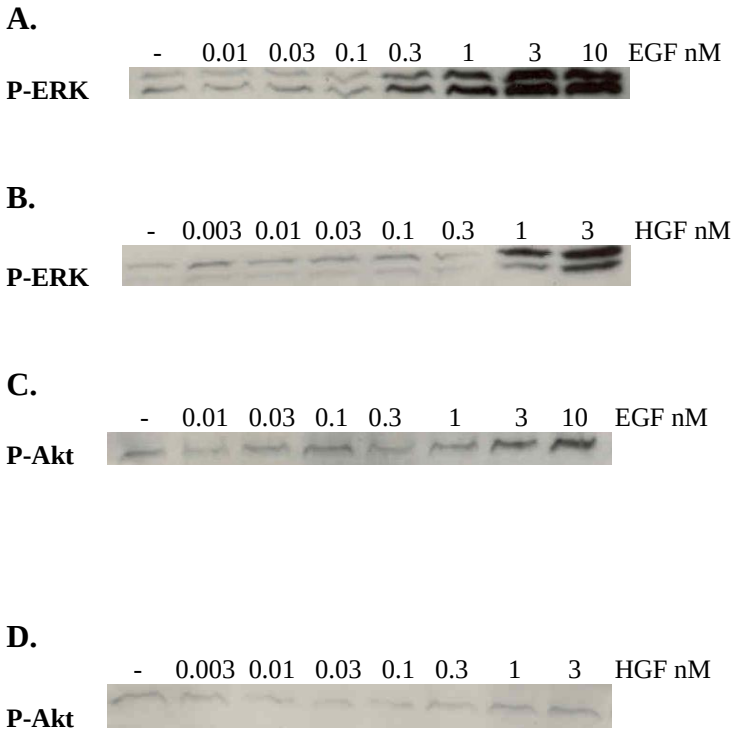


Figure 3.1 Effect of EGF induction on (A) ERK phosphorylation, (C) Akt phosphorylation and HGF induction on (B) ERK phosphorylation, (D) Akt phosphorylation

Primary rat hepatocytes were cultured in WME supplemented with 10% FCS after plating for 4h, followed with 24h SF incubation. Cells were then stimulated by different concentrations of EGF (from 10pM to 10nM) or HGF (from 3pM to 3nM) for 20min as indicated before western blotting. Western blots are representative of 3 independent experiments.

3.2.2 The effect of EGF on primary rat hepatocytes

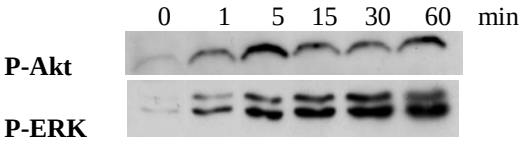
3.2.2.1 The effect of ITS on the phosphorylation of Akt and ERK

In order to improve hepatocyte survival after plating, ITS was supplied for the first 4h of incubation. However, ITS contains insulin which has been reported to induce Akt and ERK phosphorylation in hepatocytes (Peak *et al*., 1998; Senn *et al*., 2002). Therefore the effect of ITS on the phosphorylation of Akt and ERK was investigated in our cells. Cells were cultured for 4h in FCS (no ITS), then maintained serum free for 24h after which they were stimulated with ITS. The effect on Akt and ERK phosphorylation was measured following the stimulation.

Figure 3.2 shows that ITS has a potent effect on the increase of both Akt and ERK phosphorylation in primary rat hepatocytes. ITS stimulation of the phosphorylation of Akt rapidly increased and peaked at 5min. The response was maintained at a high level for about 2h before it gradually decreased over the following 22h. ITS stimulation of ERK phosphorylation gradually increased in the first 1h and maintain in a high level for over 24h.

Therefore, in future experiments, after incubating cells with ITS for 4h, ITS was removed by changing medium to SF WME and incubated for a further 48h to remove ITS induced phosphorylation of Akt and ERK.

A.



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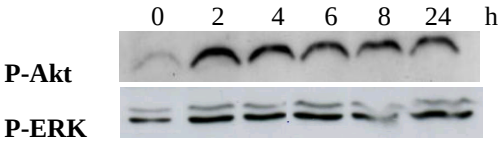


Figure 3.2 ITS stimulation of phospho-Akt and phospho-ERK A. short time course and B. long time course

Primary rat hepatocytes were cultured in WME supplemented with 10% FCS after plating for 4h, followed with 24h SF incubation. Cells were then stimulated with ITS at different time points as indicated. Western blotting for phospho-Akt and phospho-ERK were measured. Western blots are representative of 3 independent experiments.

3.2.2.2 EGF stimulation of the phosphorylation of Akt and ERK time course

The next experiment was designed to examine the extent and duration of the EGF stimulation of the phosphorylation of Akt, ERK and EGFR in hepatocytes. As shown in Figure 3.3A, EGF induced a rapid and brief phosphorylation of Akt response in hepatocytes. After exposure of cells to EGF for 1min, an increase in phospho-Akt was detected. The phosphorylation of Akt rapidly rose to peak at 5min, after which the response decreased and almost disappeared in the following 1-2h. There was little difference detected with treatment longer than 2h. EGF stimulation of phospho-ERK was slower but more stable than phospho-Akt, as shown in Figure 3.3B. The response peaked around 1h. Then the level of the phosphorylated ERK gradually decreased and almost disappeared after 4h incubation. Figure 3.3C presents EGF induced EGFR auto-phosphorylation of tyrosine kinase over time. EGF activated its receptor after exposure to cells within 1min and peaked at about 30min. This was rapidly increased and maintained for about 2h before it gradually decreased over the following 6h.

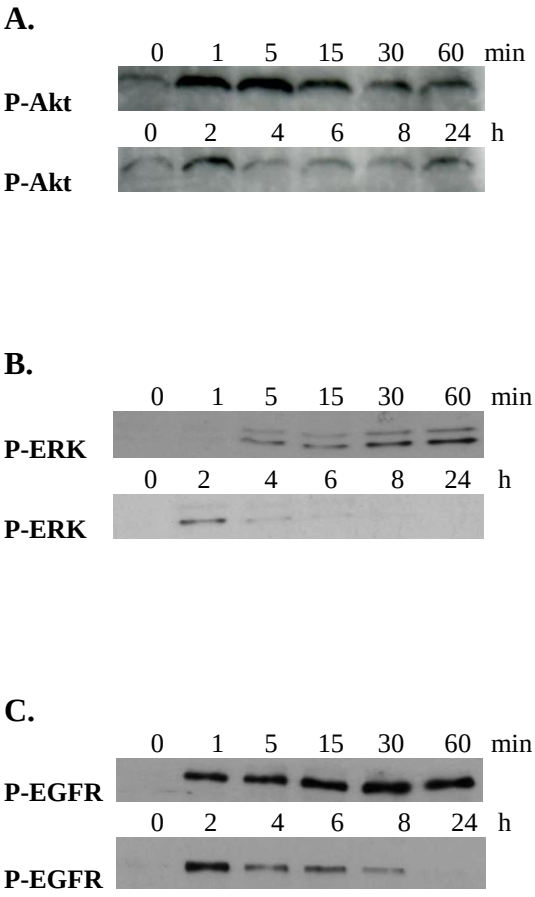


Figure 3.3 Time course of EGF stimulation of A. phospho-Akt, B. phospho-ERK and C. phospho-EGFR

Primary rat hepatocytes were cultured in WME supplemented with ITS and 10% FCS after plating for 4h, followed with 48h serum free incubation. Cells were then stimulated with 3nM EGF at different time points as indicated and western blotting for phospho-Akt and phospho-ERK. Western blots are representative of 3 independent experiments.

3.2.2.3 The effect of EGF on hepatocyte DNA synthesis and cell viability

It has previously shown that EGF can stimulate the incorporation of [^{3}H]-thymidine into DNA in the culture of rat hepatocytes (McGowan *et al*., 1981). Here we investigated the time course and concentration dependence of EGF stimulation of cell cycle progression in hepatocytes. Furthermore, the effect of EGF on cell viability was studied.

Figure 3.4 shows the results from an experiment in which hepatocytes were treated with 3nM EGF for different periods before the agonist was removed and cells were further incubated, to give a total of 24h incubation. The result demonstrated that 4h exposure of primary rat hepatocytes to EGF was the minimum time required to give a significant increase in [^{3}H]-thymidine incorporation. A treatment of 24h with EGF resulted in the largest stimulation of DNA synthesis compared to control cells. Therefore 24h exposure of the cells to EGF was used as a standard time in the rest study of ligand induction of hepatocyte DNA synthesis.

Figure 3.5 illustrates the effect of different concentrations of EGF on hepatocyte DNA synthesis and cell viability. This shows that 1nM was the lowest concentration to produce a significant stimulation of hepatocyte DNA synthesis (Figure 3.5A). The stimulation reached a maximum at 3nM and no further increase from higher concentration of EGF (10nM). However, 24h stimulation with 3nM or 10nM EGF did not result in an increase of the cell viability, as presented in Figure 3.5B.

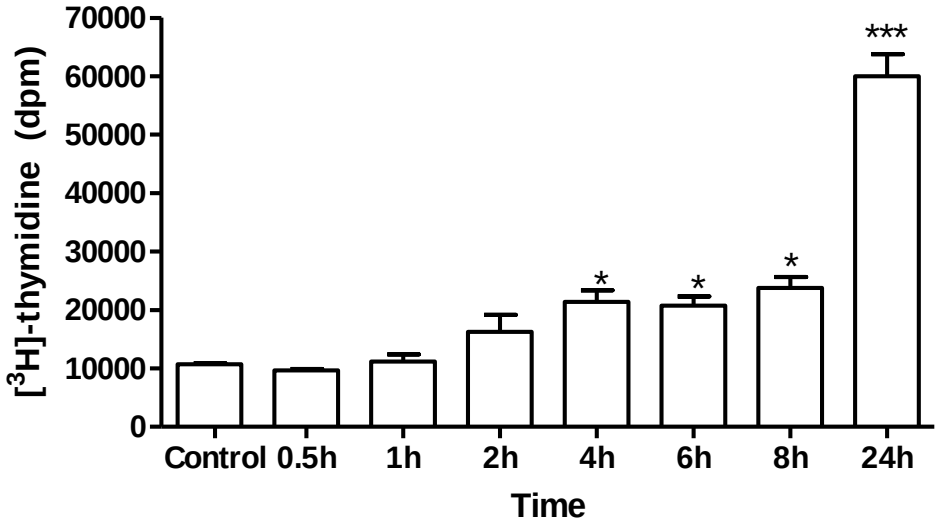
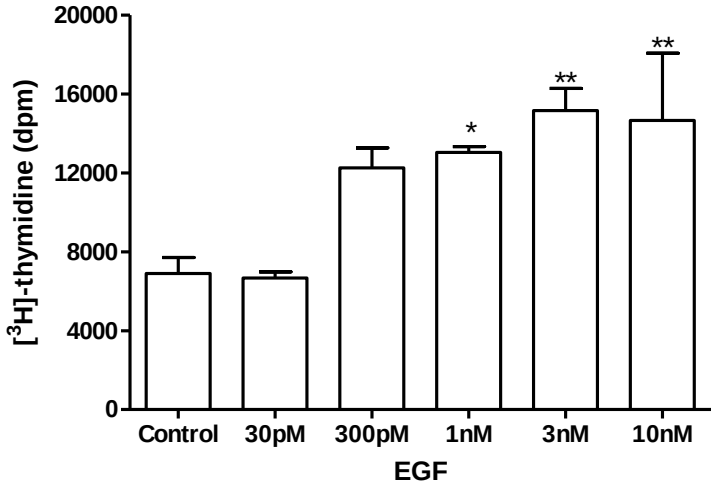


Figure 3.4 EGF stimulation of hepatocyte DNA synthesis time course

Hepatocytes were incubated in WME supplemented with ITS and 10% FCS for 4h after plating. Subsequently, cells were changed to SF WME and incubated for 24h. 3nM EGF was added and removed at different time points as indicated. All the cells were incubated 24h from the time of adding EGF. DNA synthesis was measured by [^{3}H]-thymidine assay (as described in Chapter Two, Section 2.2.2). Data represented mean ± SEM (n=4) from a single experiment and were analysed by One-way ANOVA and post test was performed with Dunnet's to compare all data sets to the control using Graphpad Prism (*$p<0.05$, ***$p<0.001$). Results are representative of 2 independent experiments..

A.



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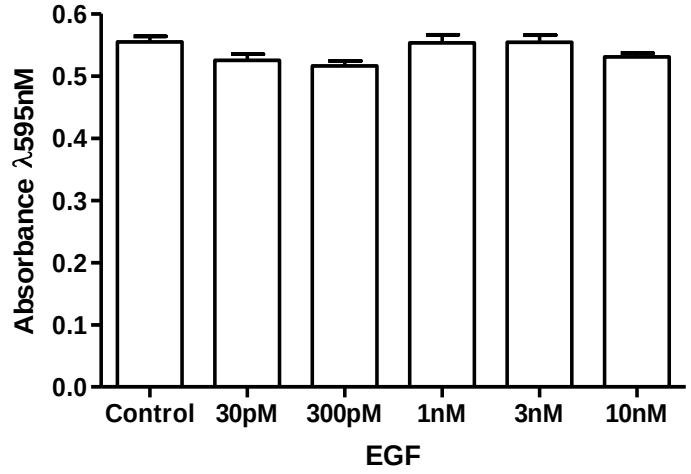


Figure 3.5 Effect of different concentrations of EGF on hepatocytes A. DNA synthesis and B. cell viability

Hepatocytes were incubated in WME supplemented with ITS and 10% FCS for 4h after plating. Subsequently cells were changed to SF WME and incubated for 24h. Different concentrations of EGF was added to the cells and left for 24h. (A) DNA synthesis was measured by [^{3}H]-thymidine assay and (B) cell viability was measured by MTT assay (as described in Chapter Two, Section 2.2.2 and 2.2.5). Data represented mean ± SEM (n=4) from a single experiment and were analysed by One-way ANOVA and post test was performed with Dunnet's to compare all data sets to the control using Graphpad Prism (*$p<0.05$, **$p<0.1$). Results are representative of 2 independent experiments.

3.2.3 The role of PI3K/Akt signalling pathway in the EGF response of primary rat hepatocytes

The previous results have shown that the level of Akt phosphorylation is increased by EGF. Here we set out to test the hypothesis that EGF stimulation of cell cycle progression requires activation of the PI3K/Akt pathway. EGF stimulation of [^{3}H]-thymidine incorporation into DNA was measured while blocking PI3K, Akt or mTOR activity. Also the phosphorylation of Akt and GSK-3 were investigated to monitor the extent of the inhibition of their up-regulated protein PI3K and Akt respectively.

3.2.3.1 The effect of PI3K on EGF stimulation

Different concentrations of LY294002 (from 1 to 20μM) were added before exposure of cells to EGF. The phosphorylation responses of Akt and GSK-3, and DNA synthesis were investigated by western blotting and [^{3}H]-thymidine assay respectively. Since DMSO was the diluent of inhibitors in most experiments, in this preliminary experiment the effect DMSO was determined.

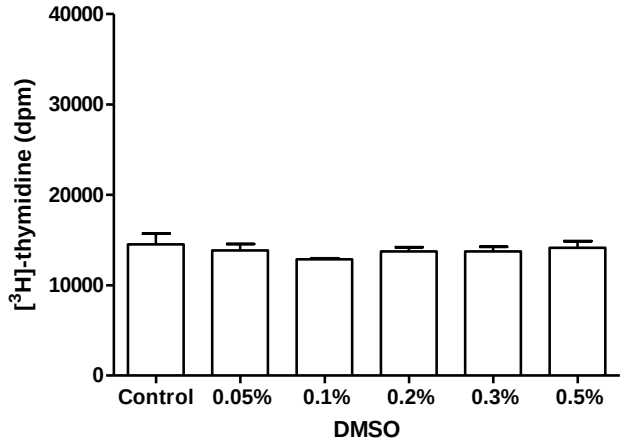
Figure 3.6A shows that up to 0.5%, DMSO did not have an effect on hepatocyte cell cycle. Furthermore, DMSO (from 0.05% to 0.5%) did not have a statistically significant effect on the EGF stimulation of [^{3}H]-thymidine incorporation into DNA as shown in Figure 3.6B. Therefore, 0.05%-0.1% of DMSO was used as the standard dilution for inhibitors in all the experiments.

Figure 3.7 illustrates that LY294002 strongly inhibited the EGF stimulation of Akt phosphorylation, while LY294002 itself did not induce the phosphorylation of Akt.

10μM LY294002 was the lowest concentration that gave a total inhibition of EGF-induced Akt phosphorylation.

Figure 3.8 demonstrates that neither LY294002 nor its solvent (0.1% DMSO) significantly reduced hepatocyte cell viability in 24h incubation. It also can be seen from Figure 3.9 that 10μM LY294002 totally inhibited EGF-induced DNA synthesis and significantly reduced [^{3}H]-thymidine incorporation into DNA in un-stimulated hepatocytes. This indicates that the decrease of DNA synthesis resulting by LY294002 was not due to cell death but because of inhibition of Akt activation.

A



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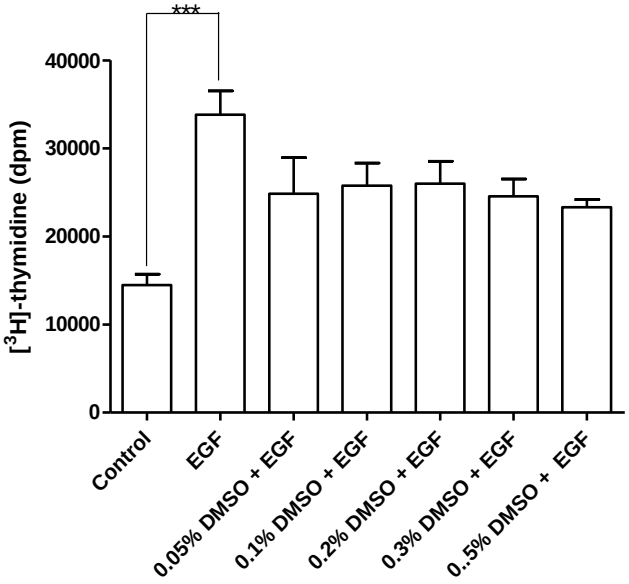


Figure 3.6 Effect of increasing concentrations of DMSO on A. un-stimulated and B.EGF stimulated hepatocytes

Hepatocytes were cultured in WME supplemented with ITS and 10% FCS for 4h after plating and then further incubated in SF WME for 24h. Different concentrations of DMSO were added 15min before incubation of cells with/without EGF for 24h. DNA synthesis was measured by [^{3}H]-thymidine assay (as described in Chapter Two, Section 2.2.2). Data represented mean ± SEM (n=4) from a single experiment and were analysed by One-way ANOVA and post test was measured with Bonferroni's to compare all data sets using Graphpad Prism (***$p<0.001$). Results are representative of 2 independent experiments.

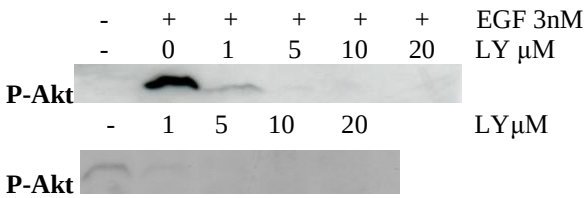


Figure 3.7 Effect of different concentrations of LY294002 on EGF-induced Akt phosphorylation

Hepatocytes were incubated in WME supplemented with ITS and 10% FCS for 4h after plating followed with 48h SF incubation before stimulation. Cells were pre-incubated with different concentrations of LY294002 for 15min before incubation with/without 3nM EGF for 5min. Phospho-Akt was measured by western blotting. Western blots are representative of 3 independent experiments.

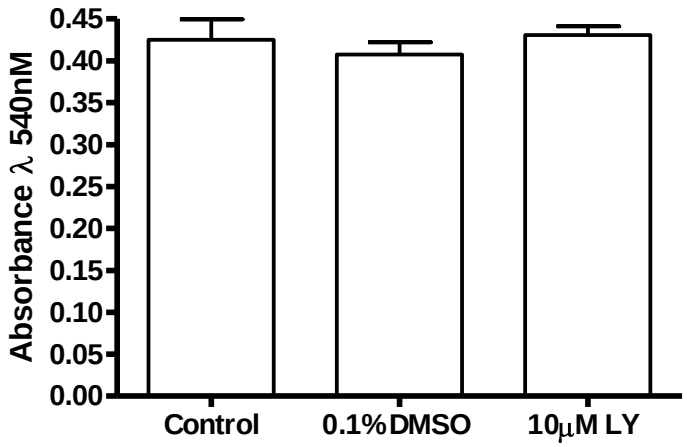


Figure 3.8 Effect of DMSO and LY294002 on hepatocyte viability

Hepatocytes were cultured in WME supplemented with ITS and 10% FCS for 4h after plating and then further incubated in SF WME for 24h. DMSO or LY294002 was added 15min before incubation of cells with/without EGF for 24h. Cell viability was measured by MTT assay (as described in Chapter Two, Section 2.2.5). Data represented mean ± SEM (n=4) from a single experiment and were analysed by One-way ANOVA using Graphpad Prism (no significant difference). Results are representative of 3 independent experiments.

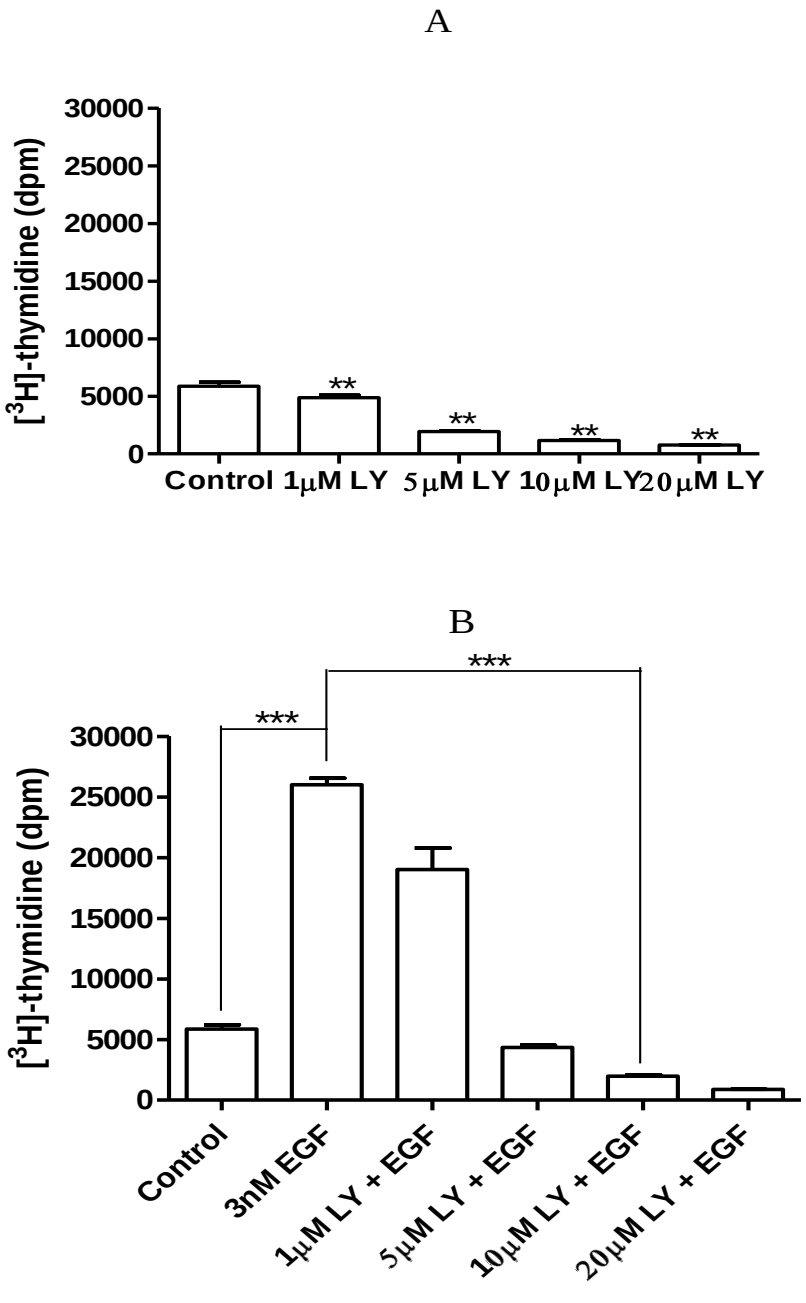


Figure 3.9 Effect of different concentrations of LY294002 on A. un-stimulated and B. EGF-stimulated hepatocyte DNA synthesis

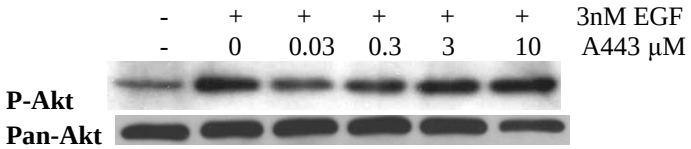
Hepatocytes were cultured in WME supplemented with ITS and 10% FCS for 4h after plating and then further incubated in SF WME for 24h. Different concentrations of LY294002 were added 15min before incubation of cells with/without EGF for 24h. DNA synthesis was measured by [^{3}H]-thymidine assay. Data represented mean ± SEM (n=4) from a single experiment and were analysed by One-way ANOVA and post test was measured with Bonferroni's to compare all data sets using Graphpad Prism (**p<0.1, ***p<0.001). Results are representative of 3 independent experiments.

3.2.3.2 The effect of Akt on EGF stimulation

Luo and colleagues have reported that A443654 is a selective inhibitor of Akt (Luo *et al*., 2005). Here we investigated whether in hepatocytes, A443654 inhibits EGF stimulation of Akt phosphorylation and/or activity of Akt. The phosphorylation of Akt and its substrate (GSK-3) was measured using western blotting. If A443654 is a selective inhibitor of Akt activity, then it should not affect the phosphorylation of Akt but it should inhibit GSK-3 phosphorylation.

Figure 3.10A shows that A443654 at concentrations up to 10μM did not inhibit the EGF induction of Akt phosphorylation. However, 3μM A443654 completely abolished EGF-stimulated GSK-3 phosphorylation. Inhibition started at a concentration of 30nM, the lowest concentration used (Figure 3.10B). It was then able to use A443654 to investigate the dependence of the EGF stimulation of the cell cycle on Akt. The influence of different concentrations of A443654 on EGF stimulated thymidine incorporation is shown in Figure 3.11. At 3μM, A443654 significantly inhibited ($p<0.001$) the EGF stimulated DNA synthesis. Consequently, 3μM A443654 was used in further experiments to block Akt activity.

A



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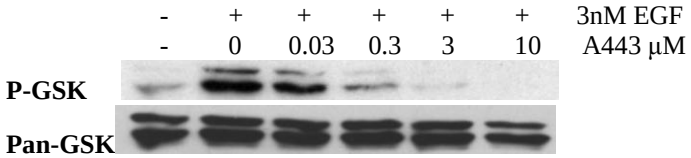


Figure 3.10 Effect of different concentrations of A443654 on EGF-induced A. phospho-Akt and B.phospho- GSK

Hepatocytes were incubated in WME supplemented with ITS and 10% FCS for 4h after plating followed by 48h SF incubation before stimulation. Cells were pre-incubated with different concentrations of A443654 for 30min before incubation with/without 3nM EGF for 5min. Phospho-Akt and phospho-GSK were measured by western blotting (as described in Chapter Two, Section 2.2.3). Western blots are representative of 3 independent experiments.

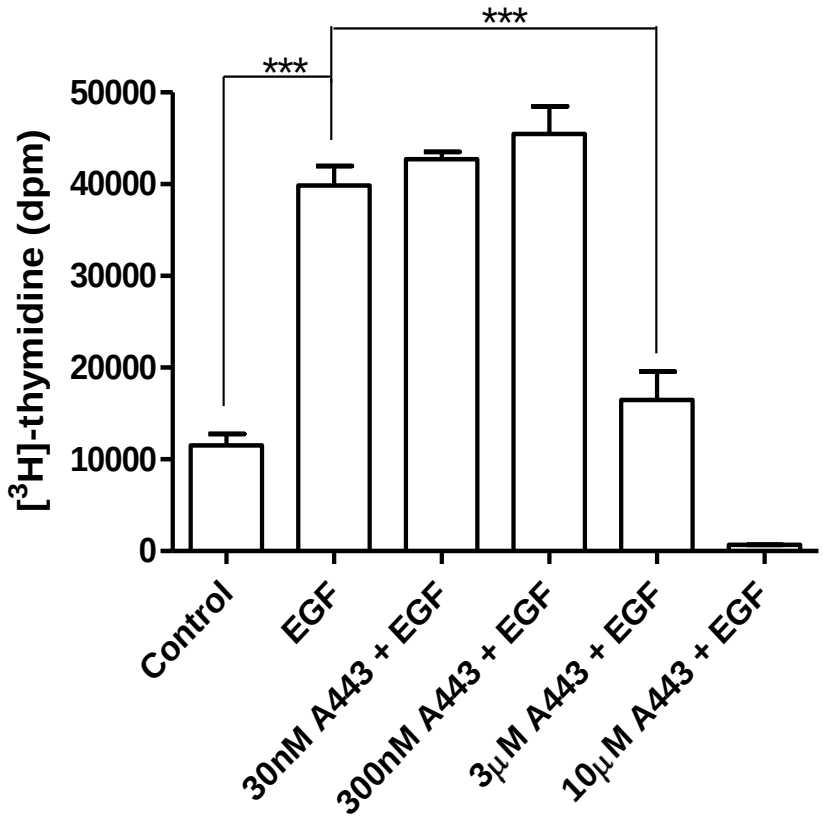


Figure 3.11 Effect of different concentrations of A443654 on the EGF stimulation of hepatocyte DNA synthesis

Hepatocytes were cultured in WME supplemented with ITS and 10% FCS for 4h after plating and then further incubated in SF WME for 24h. Different concentrations of A443654 werr added 30min before exposure of cells to EGF for 24h. DNA synthesis was measured by [^{3}H]-thymidine assay. Data represented mean ± SEM (n=4) from a single experiment and were analysed by One-way ANOVA and post test was performed with Bonferroni's to compare all data sets using Graphpad Prism (***$p<0.001$). Results are representative of 3 independent experiments.

3.2.3.3 The effect of Akt on hepatocyte cell cycle progression and cell viability

The previous work has shown that Akt is necessary for the cell cycle response to EGF using PI3K and Akt inhibitors. The role of Akt was investigated further using adenovirus expressing dominant-negative (Dn) Akt and constitutively active (myr) Akt. The experimental work was carried out by collaboration with Dr John Hall and Mr Zhong Cheng.

The optimal time for exposure of hepatocytes to adenovirus encoding Dn-Akt and myr-Akt was determined by treating the cells for 2h, 16h and 48h as shown in Figure 3.12. In the experiment, HEK-293 cell lysis solution containing Dn-Akt (4μl) or myr-Akt (1μl) encoded adnovirous was added to 3ml of $1x10^5$ hepatocytes. The results show that, following exposure of cells to 1:4 (volume/volume) of HEK-293 cell lysis solution contacting myr-Akt and Dn-Akt encoded adenovirus, hepatocytes presented with a similar level of Akt expression. The 16h exposure gave the strangest level of the expression of Akt (Dn and myr) in hepatocytes. Furthermore, long exposure (48h) of hepatocytes to adenovirus caused the formation of groups of cells see under mircoscope (data not shown). Therefore, cells were exposed to adenovirus for 2h/16h in the beginning of 24h/48h incubation before stimulating cells with ligand such as EGF in following experiments. Threrfore, in further experiments, cells were cultured with 10% FCS and ITS for 4h as usual following 2h/16h exposure of the cells to adenovirus encoding Dn-Akt and myr-Akt (4/1 v/v). Adenovirus was removed by changing to SF medium and cells were further incubated 22h/32h to allow the expression of mutant Akt before stimulation.

In order to investigate the role of Akt in the EGF stimulation of cell cycle progression, Dn-Akt and myr-Akt were incorporated into hepatocytes by adenovirus infection. Adenovirus was incubated with cells for 2h to allow the RNA replication and cells were further incubated for 22h without adenovirus to produce target proteins. Cells were exposed to EGF for 24h and DNA synthesis and cell viability were measured to investigate the role of Akt in the un-stimulated cell cycle, and EGF-stimulated cell cycle progression.

The results in Figure 3.13A show that Dn-Akt significantly inhibited the EGF stimulation of hepatocyte DNA synthesis and myr-Akt increased the level of [^{3}H]-thymidine incorporation into DNA. Furthermore EGF significantly increased the level of [^{3}H]-thymidine incorporation into DNA in myr-Akt expressing hepatocytes. Dn-Akt reduced EGF stimulation of DNA synthesis to the control level. Interestingly, as shown in Figure 3.13B, myr-Akt caused a statistically significant increase in the MTT measure of cell viability. However, addition of EGF did not increase cell viability in either normal hepatocytes or in the myr-expressing cells. Importantly, there was no change in cell viability with the Dn-Akt treated cells. This indicated that the inhibition of Akt activity did not directly cause a decrease of the cell viability.

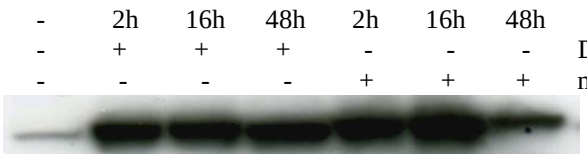


Figure 3.12 Dn-Akt and myr-Akt expression time course in primary rat hepatocyte

Hepatocytes were cultured for 4h in WME supplemented with ITS and 10% FCS, which were then replaced with SF WME containing encoding kinase-dead Dn-Akt or constitutively active myr-Akt. At different time points as described adenovirus were removed by changing the medium and cells were further incubated for a total of 24h incubation before Pan-Akt was measured by western blotting (at 48h, adenovirus was left for all 48h period). Western blots are representative of 3 independent experiments. Blots are provided by Mr Zhong Cheng.

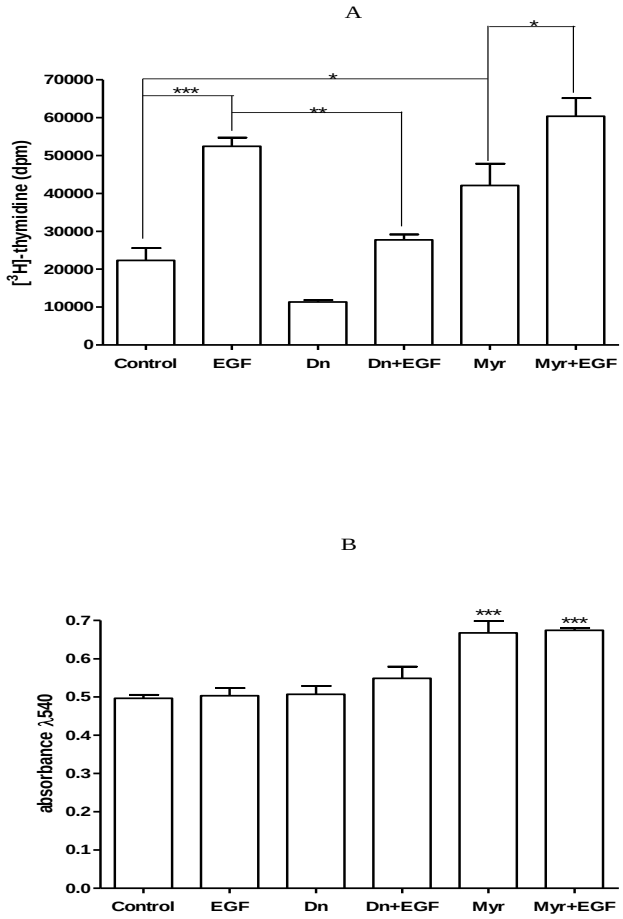


Figure 3.13 Effect of Akt on hepatocyte A. DNA synthesis and B. cell viability

Hepatocytes were cultured for 4h in WME supplemented with ITS and 10% FCS, which were then replaced with SF WME containing encoding kinase-dead Dn-Akt or constitutively active myr-Akt for 2h and further incubated for 22h before supplement with/without 3nM EGF for another 24h. [^{3}H]-thymidine incorporation and cell viability were measured by thymidine assay and MTT assay, respectively. Data represented mean ± SEM (n=4) from a single experiment and were analysed by One-way ANOVA and post test was performed with Bonferroni's to compare all data sets (A) and Dunnet's to compare all data sets to the control (B) using Graphpad Prism(*$p<0.05$, **$p<0.1$, ***$p<0.001$). Results are representative of 3 independent experiments. Data are provided by Dr John Hall.

3.2.3.4 The effect of mTOR on hepatocyte cell cycle progression

The activity of mTOR has been reported to be regulated by Akt (Nave *et al*., 1999). Furthermore, Coutant *et al* (2002) reported that mTOR was critical for the EGF stimulation of cell proliferation in hepatocytes. Therefore, here the role of mTOR in EGF stimulation of cell cycle progression in our cells was investigated using its inhibitor-rapamycin (Rap).

Figure 3.14 shows that 3nM rapamycin completely abolished EGF stimulation of [^{3}H]-thymidine incorporation into DNA in our cells.

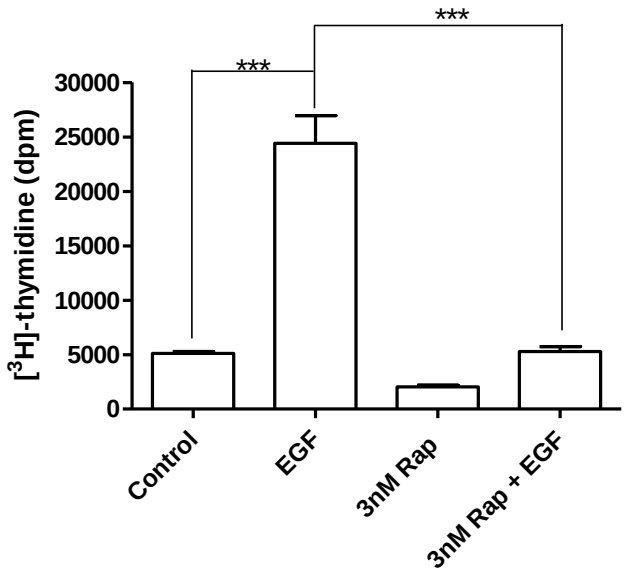


Figure 3.14 Effect of mTOR on hepatocyte DNA synthesis

Hepatocytes were cultured in WME supplemented with ITS and 10% FCS for 4h after plating and then further incubated in SF WME for 24h. 3nM Rap was added 30min before stimulation of cell with 3nM EGF for 24h. DNA synthesis was measured by [^{3}H]-thymidine assay. Data represented mean ± SEM (n=4) from a single experiment and were analysed by One-way ANOVA and post test was performed with Bonferroni's to compare all data sets using Graphpad Prism (***$p<0.001$). Results are representative of 3 independent experiments.

3.2.4 The role of MEK/ERK signalling pathway in EGF stimulation of primary rat hepatocytes

The previous results demonstrated that the level of the phosphorylation of ERK was increased after the exposure of cells to EGF. This indicated that ERK may play a crucial role in the cell response to EGF stimulation. To investigate the role of ERK in EGF stimulation, the activity of ERK was inhibited by blocking its upstream protein kinase-MEK activity with UO126. The EGF stimulation of cell cycle progression was then measured by [^{3}H]-thymidine assay. A UO126 concentration-response experiment was firstly performed to find a suitable concentration which totally inhibited the EGF stimulation of ERK phosphorylation.

It can be seen from Figure 3.15 that 10μM UO126 was the lowest concentration tested that completely abolished the EGF triggered phosphorylation of ERK. However, as shown in Figure 3.16, the inhibition of the phosphorylation of ERK did not result in a complete inhibition of the EGF stimulation of hepatocyte DNA synthesis. Although 10μM UO126 significantly reduced the EGF stimulation of hepatocyte DNA synthesis, the level of [^{3}H]-thymidine incorporation was still significantly greater than in un-stimulated control cells.

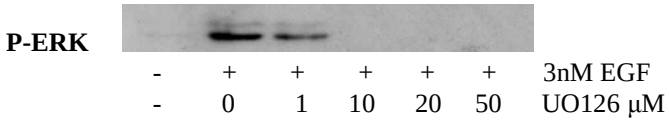


Figure 3.15 Inhibition of the EGF induced phospho-ERK in hepatocytes by different concentrations of UO126

Hepatocytes were incubated in WME supplemented with ITS and 10% FCS for 4h after plating followed with 48h S.F incubation before stimulation. Cells were pre-incubated with different concentrations of UO126 for 15min before incubated with/without 3nM EGF for 20min. Phospho-ERK was measured by western blotting. Western blots are representative of 3 independent experiments.

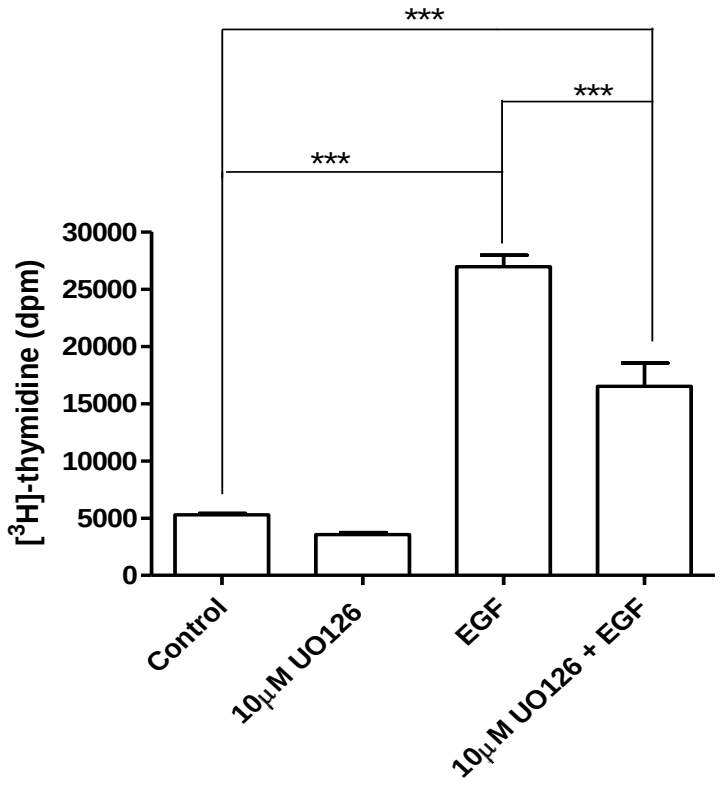


Figure 3.16 Effect of 10μM UO126 on the EGF stimulation of hepatocyte DNA synthesis

Hepatocytes were cultured with 10% FCS and ITS for 4h after plating and then further incubated in SF WME for 24h. 10μM UO126 was added 15min before incubation of cells with/without EGF for 24h, as indicated. DNA synthesis was measured by [^{3}H]-thymidine assay. Data represented mean ± SEM (n=4) from a single experiment and were analysed by One-way ANOVA and post test was performed with Bonferroni's to compare all data sets using Graphpad Prism (***$p<0.001$). Results are representative of 3 independent experiments.

3.2.5 The role of tyrosine kinase in EGF stimulation of primary rat hepatocytes

In order to investigate whether EGF stimulating the auto-phosphorylation of its receptor and hepatocyte DNA synthesis were due to EGF inducing EGFR/EGFR homo-dimerisation or EGFR/ErbB2 hetero-dimerisation, AG1478 and AG825 were used to inhibit the homo- and hetero- dimerisation dependent tyrosine kinase phosphorylation of the receptor respectively. The EGF induction of the EGFR phosphorylation and [^{3}H]-thymidine incorporation into DNA were measured.

3.2.5.1 The effect of AG1478 on EGF stimulation

The results presented in Figure 3.17 show that increasing concentrations of AG1478 inhibited the EGF stimulation of EGFR phosphorylation. 300nM was the lowest concentration at which AG1478 almost completely abolished the EGF-induced EGFR phosphorylation. Also as shown in Figure 3.18, this concentration of AG1478 also succeeded in a significant inhibition of EGF stimulation of [^{3}H]-thymidine incorporation into DNA.

3.2.5.2 The effect of AG825 on EGF stimulation

The results (Figure 3.19) show that AG825 had no effect on the phosphorylation of EGFR in either EGF-stimulated or un-stimulated cells. There was also a minimal or insignificant effect of AG825 on the EGF stimulation of hepatocyte DNA synthesis (Figure 3.20).

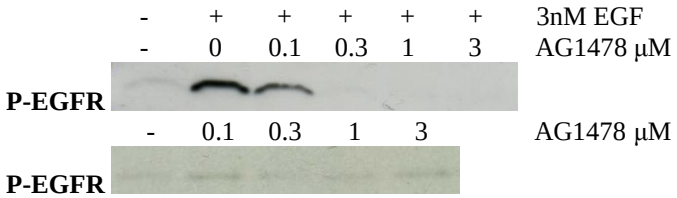


Figure 3.17 Effect of AG1478 on EGF stimulation of phosphorylation of EGFR

Hepatocytes were incubated in WME supplemented with ITS and 10% FCS for 4h after plating followed with 48h SF incubation before stimulation. Cells were pre-incubated with different concentrations of AG1478 for 15min before incubated with/without 3nM EGF for 20min. Phospho-EGFR was measured by western blotting (as described in Chapter Two, Section 2.2.3). Western blots are representative of 3 independent experiments.

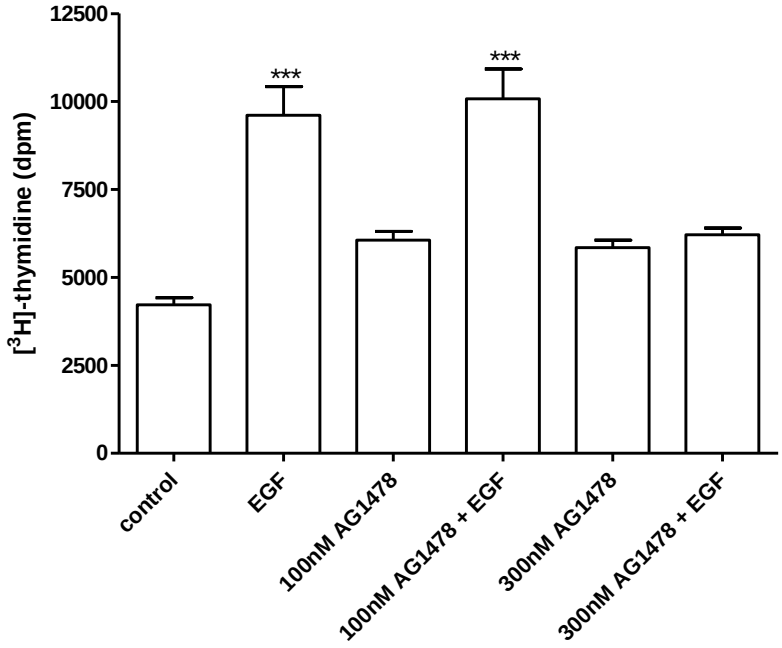


Figure 3.18 Effect of different concentrations of AG1478 on EGF stimulation of hepatocyte DNA synthesis

Hepatocytes were cultured in WME supplemented with ITS and 10% FCS for 4h after plating and then further incubated in S.F WME for 24h. Different concentrations of AG1478 were added 15min before incubation of cells with/without EGF for 24h as indicated. DNA synthesis was measured by thymidine assay (as described in Chapter Two). Data represented mean ± SEM (n=4) from a single experiment and were analysed by One-way ANOVA and post test was performed with Dunnet's to compare all data sets to the control using Graphpad Prism (***$p<0.001$). Results are representative of 3 independent experiments.

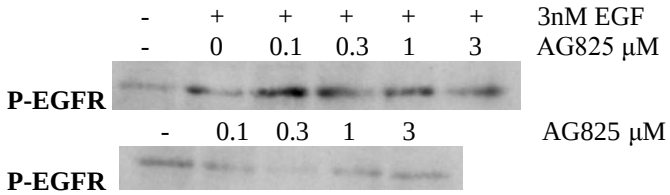


Figure 3.19 Effect of AG825 on EGF stimulation of EGFR phosphorylation

Hepatocytes were incubated in WME supplemented with ITS and 10% FCS for 4h after plating followed with 48h SF incubation before stimulation. Cells were pre-incubated with different concentrations of AG825 for 15min before incubated with/without 3nM EGF for 20min. Phospho-EGFR was measured by western blotting. Western blots are representative of 3 independent experiments.

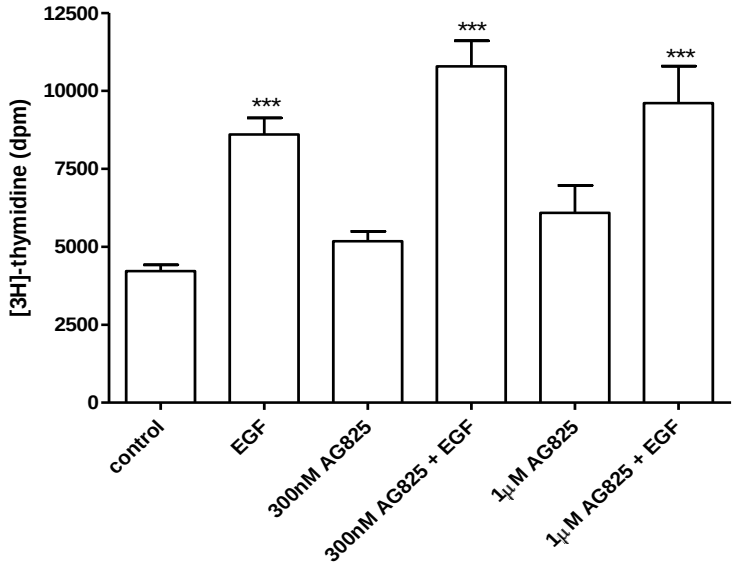


Figure 3.20 Effect of different concentrations of AG1478 on EGF stimulation of hepatocyte DNA synthesis

Hepatocytes were cultured in WME supplemented with ITS and 10% FCS for 4h after plating and then further incubated in SF WME for 24h. Different concentrations of AG825 were added 15min before incubation of cells with/without EGF for 24h, as indicated. DNA synthesis was measured by [^{3}H]-thymidine assay. Data represented mean ± SEM (n=4) from a single experiment and were analysed by One-way ANOVA and post test was performed with Dunnet's to compare all data sets to the control using Graphpad Prism (***$p<0.001$). Results are representative of 3 independent experiments.

3.3 Discussion

HGF has been reported to play a central role in liver regeneration and hepatocyte proliferation. Evidence from c-Met mutant mice (non-activated c-Met) proved that when c-Met activation was inhibited, the liver regeneration was significantly reduced after PH (Borowiak *et al*., 2004). EGF also has been reported to be important for liver regeneration and hepatocyte proliferation via the PI3K/Akt and MEK/ERKs pathway (Jones *et al*., 1995; Roberts *et al*., 2000; Coutant *et al*., 2002). The results presented here suggested that in primary rat hepatocytes, EGF produced a stronger stimulation of both the phosphorylation of Akt and ERK than the response induced by the same concentration of HGF. EGF also induced [^{3}H]-thymidine incorporation into DNA which demonstrated that EGF driving quiescent hepatocytes from G0 into S phase. This finding agreed with the work of others (Loyer *et al*., 1996). EGF did not increase the cell viability, which indicated that under the conditions of our experiment, EGF did not induce hepatocyte proliferation. However, Yoshizato and colleagues claimed that they have achieved limited hepatocyte proliferation in culture (Yamasaki *et al*., 2006). This could be due to the medium (hepatocyte clonal growth medium; HCGM) used to support a longer period of the growth of hepatocytes and/or human serum which stimulated cell proliferation in their experiments. In order to minimise cell cycle progression ([^{3}H]-thymidine response) in the control (unstimulated) cells in our experiments, cells were routinely cultured in SF WME to achieve most cells entering G0 phase before stimulation. Also, it could be because small amount of the cell proliferation could not produce a measurable effect on cell viability.

In rat hepatocytes, the PI3K/Akt/mTOR pathway has a crucial role in the cell response to EGF. This signalling pathway was vital for EGF effect since EGF stimulation of DNA synthesis was completely abolished by the inhibition of PI3K activity by the selective inhibitor LY294002. Moreover, when Akt or mTOR activity was inhibited by A443654 or rapamycin respectively, EGF-induced hepatocyte DNA synthesis was abolished. This was in partial agreement with the work of another group who showed that the PI3K/mTOR pathway was necessary for the EGF stimulation of hepatocyte proliferation (Coutant *et al*., 2002). However, the same group has also shown that blocking Akt activity by the expression of Dn-Akt did not affect EGF stimulation of cell cycle progression and in turn concluded that the activation of Akt may not be necessary in this pathway. In fact, this could be because of the low transfection efficiency (35% to 40%) in their experiment. The results presented in this chapter showed that EGF stimulation of the phosphorylation of Akt happened in the very early stage after exposure of hepatocytes to EGF (the response was detected 1min after stimulation). This indicated the crucial role that Akt may play in EGF stimulation of hepatocytes. Furthermore, only a small amount of total Akt activity was sufficient to give a complete [^{3}H]-thymidine response. It has been shown that 300nM A443654 inhibits most of the Akt activity (ie stimulation of GSK-3 phosphorylation) (Figure 3.10); however, it did not affect the level of EGF-induced [^{3}H]-thymidine incorporation into DNA (Figure 3.11). The importance of Akt in hepatocyte response to EGF stimulation was also supported by using Akt selective inhibitor. The inhibition of the activity of Akt with A443654 (3μM) leaded to a complete abolishment of EGF stimulation of [^{3}H]-thymidine incorporation into DNA (Figure 3.11). Further strong evidence that Akt is necessary for EGF stimulation of cell cycle progression came from

Dn-Akt work. When hepatocytes expressed Dn-Akt following infection of cells with encoded adenovirus, EGF failed to induce cell cycle progression. This strongly suggested that Akt plays an essential role in the cell response to EGF stimulation. Furthermore, when the cell expressed myr-Akt, there was a significant increase of the level of [^{3}H]-thymidine incorporation into DNA comparing to un-stimulated control cells. Also myr-Akt expressing cells presented a higher viability than the control cells. These suggested that the continued activation of Akt may result in DNA synthesis.

Furthermore, the expression of myr-Akt might also induce hepatocyte proliferation (cell viability response). However, the increase of cell viability in myr-Akt expressed cells might be also due to the increase of the cell survival rate, because long-term incubation of un-stimulated hepatocytes showed a decreasing curve of cell viability. Treatment of EGF further increased the level of [^{3}H]-thymidine incorporation into DNA in myr-Akt expressed hepatocytes; however, the response was not greater than in EGF-induced control hepatocyte (no myr-Akt expressed) (Figure 3.13A). It can be due to 3nM EGF induced maximum [^{3}H]-thymidine incorporation response and therefore myr-Akt expression did not increase the response. EGF did not further increase cell viability in myr-Akt expressed cells (Figure 3.13B). This suggested that continual activation of Akt may increase the cell survival which in turn results in a higher DNA synthesis rate and cell viability than in control hepatocytes (no myr-Akt expression). However, Akt did not induce cell proliferation. The results also demonstrated that EGF stimulation hepatocyte cell cycle progression was Akt dependent. Finally, inhibition of Akt activity with Dn-Akt did not reduce cell viability in EGF-stimulated or un-stimulated cells indicating that the inhibition of Akt activity does not directly lead to

apoptosis. It has been shown that EGF suppression of TGF-β mediated hepatocyte apoptosis is PI3K/Akt pathway dependent (Roberts *et al*., 2000). The result suggested that Akt may have an anti-apoptic effect.

ERK also plays an important role in the EGF stimulation of hepatocyte cell cycle progression. It was phosphorylated in the early stages after exposure of cells to EGF. Inhibition of the activity of ERK by blocking its upstream protein MEK activation led to a significant reduction in EGF stimulation of [^{3}H]-thymidine incorporation into hepatocyte DNA. However, complete inhibition of the phosphorylation of ERK did not result in abolishment of EGF induction of DNA synthesis. This result did not correspond to the work of Baffet and colleagues, who showed that MEK/ERK was necessary for EGF stimulation of hepatocyte cell cycle progression (Coutant *et al*., 2002). This might be due to the higher concentration of MEK inhibitor-UO126 (50μM) they used in their experiment. The results in this chapter showed that 10μM UO126 completely inhibited EGF-stimulated ERK phosphorylation but only partially inhibited EGF-stimulated cell cycle progression to S phase. This suggested that other pathways, insensitive to UO126, lead to EGF stimulation of the cell cycle. For instance, JNK has been reported to be involved in the regulation of hepatocytes response to growth factor inducing cell cycle progression (Auer *et al*., 1998). Alternatively, it could be that 10μM UO126 did not actually completely inhibit ERK activity but reduced the phosphorylation of ERK to levels below the detection limit of western blotting. In conclusion, the results presented in this chapter suggested that ERK was important but may be not necessary for all EGF-stimulated cell cycle progression in hepatocytes. Furthermore, the inhibition of ERK activity did not lead to a significant decrease of

DNA synthesis in un-stimulated hepatocytes, which indicated that ERK may be more important to hepatocyte proliferation rather than survival.

Since the EGFR is a member of the superfamily-RTKs, tyrosine kinase plays a crucial role in EGF stimulation pathway (Thompson and Gill, 1985; Wells, 1999). Inhibition of the ligand-induced auto-phosphorylation of tyrosine kinase on the EGFR CT domain caused the abolishment of EGF stimulating the phosphorylation of any downstream proteins on its signalling pathway and cell cycle progression. Furthermore, in primary rat hepatocytes, binding with EGF leaded to a homo-dimerisation of EGF-receptor rather than hetero-dimerisation, since AG825 did not inhibit EGF-induced auto-phosphorylation of EGFR. It has been reported that the stimulation of EGFR/ErbB2 hetero-dimerisation may be more stable and stronger than EGFR homo-dimerisation because the hetero-dimer complexes are more stable at the cell surface and prefer recycling back to the cell surface and re-activating after internalisation, whereas the homo-dimers are usually undergoing degradation after internalisation (Levkowitz *et al*., 1998). The results in this chapter showed that EGF mainly inducing EGFR homo-dimerisation rather than hetero-dimerisation in cultured hepatocytes. This could be one of the possibilities explaining why EGF failed to induce cultured hepatocyte proliferation.

Chapter 4

The Effect of Nucleotides on Proliferation-Related Response in Primary Rat Hepatocytes

4.1 Introduction

It has been shown that exposure of primary rat hepatocytes to extracellular ATPγS, a nonhydrolysable ATP analog, activated early response genes such as c-jun and c-fos expression of which is essential for the cell to enter S phase (Thevananther *et al*., 2004). Also, it has been reported that in hepatocytes, excellular ATP and UTP stimulated cell proliferation-associated responses such as increased cytosolic free Ca^{2+} concentration and the phosphorylation of ERK and JNK (Dixon *et al*., 2005). Furthermore, extracellular ATP initiated JNK signalling in regenerating liver and significantly increased the rate of liver regeneration after PH *in vivo* (Thevananther *et al*., 2004; Kerem *et al*., 2006). These reports suggested that extracellular nucleotides may play an important role in liver regeneration and hepatocyte proliferation. Therefore, the effect of a range of nucleotides including ATP, UTP, UDP, 2MesADP and ADPβS on some proliferation-related responses of rat hepatocytes were studied. Moreover, these nucleotides were chosen to cover the majority of rat hepatocyte subtypes of P2Y receptors. Functional $P2Y_1$, which has been identified in rat hepatocytes, can be activated by 2MesADP (Dixon *et al*., 2000). ATP and UTP are the agonists of the $P2Y_2$ receptor, which has been shown to express functional subtype in rat hepatocytes (Dixon *et al*., 2000). Furthermore, it has been shown that the transcripts for $P2Y_4$, $P2Y_6$, $P2Y_{12}$ and $P2Y_{13}$ were expressed in freshly isolated rat hepatocytes (Dixon *et al*., 2000; Dixon *et al*., 2004). Therefore, UTP and ATP which are potent activators for $P2Y_4$ (Bogdanov 1998), $P2Y_6$ receptor agonist UDP (Chang *et al*., 1995), 2MesADP the most potent agonist for $P2Y_{12}$ (Unterberger *et al*., 2002) and $P2Y_{13}$ receptor agonist ADPβS and 2MesADP (Marteau *et al*., 2003) have been chosen. In order to determine the effect of each nucleotide on the proliferation-related response in rat hepatocytes, the

phosphorylation of Akt and ERK, [^{3}H]-thymidine incorporation into DNA and cell viability were investigated.

4.2 Results

4.2.1 Comparison of different types of nucleotides on the proliferation-related response

4.2.1.1 The effect of nucleotides on the induction of the phosphorylation of Akt and ERK in hepatocytes

In order to compare the ability of each nucleotide/P2Y receptor effect on rat hepatocyte proliferation, nucleotide stimulation of the phosphorylation of Akt and ERK was investigated by western blotting. Cells were exposed to the same concentration of nucleotides (100μM) for 5min and 20min before western blotting.

In cultured rat hepatocytes, it has found that ATP and UTP stimulated Akt and ERK phosphorylation with the ATP stimulation being greater than UTP, as shown in Figure 4.1. ADPβS also induced the phosphorylation of Akt and ERK while 2MesADP appeared to stimulate ERK phosphorylation. The other nucleotides tested did not produce a considerable response.

4.2.1.2 Nucleotide effects on the regulation of cell cycle and proliferation in hepatocytes

Figure 4.2A shows ATP slightly increased the level of [^{3}H]-thymidine incorporation into DNA, while treatment with 2MesADP inhibited DNA synthesis in primary rat hepatocytes. Moreover, although it was not statistically significant, ADPβS also increased the DNA synthesis response. All the treatments of nucleotides appeared to reduce hepatocytes viability, although UTP did not statistically significantly decrease the response (Figure 4.2B).

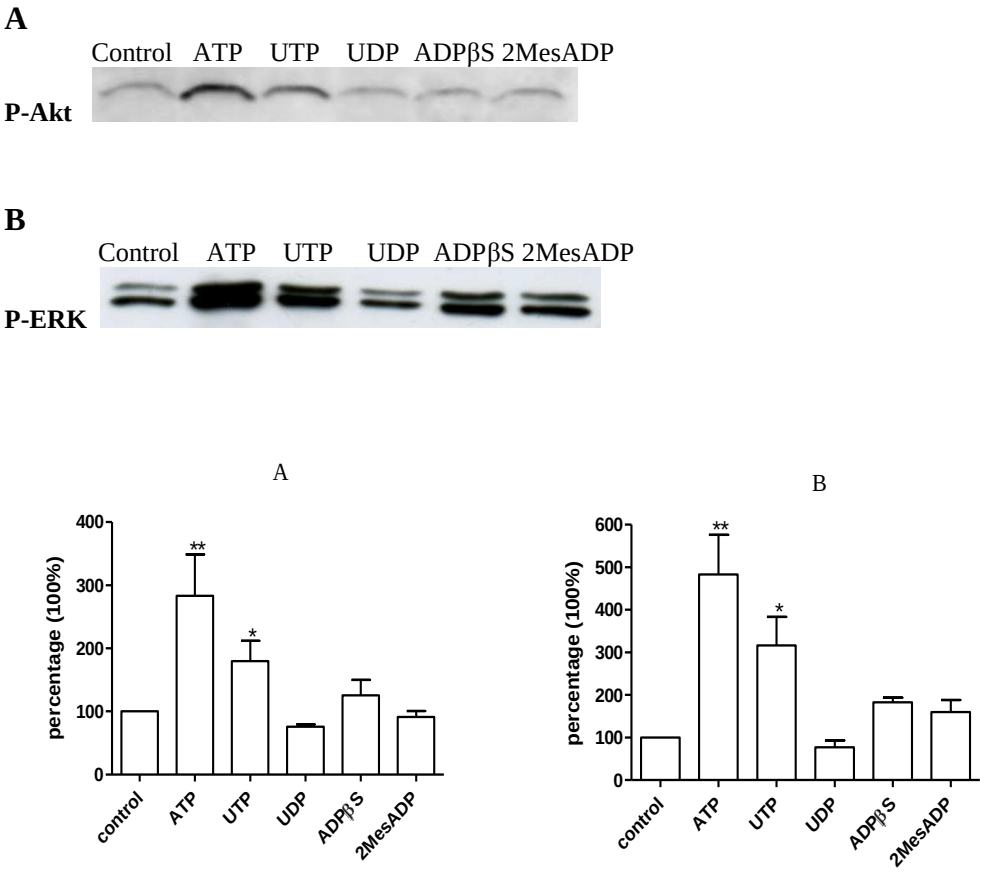
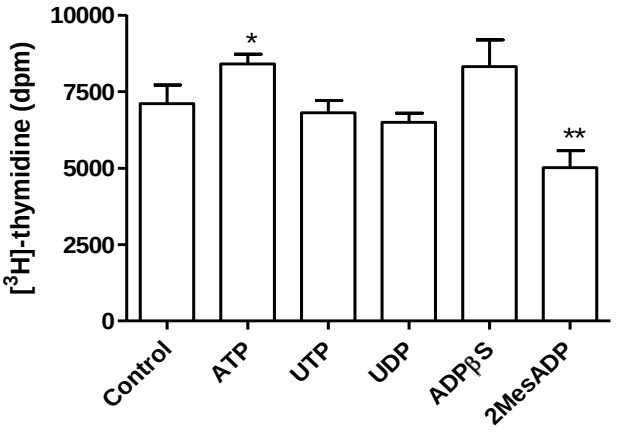


Figure 4.1 Comparison the effect of each nucleotide on the induction of A. phospho-Akt and B. phospho-ERK

Primary rat hepatocytes were cultured in WME supplemented with ITS and 10% FCS after plating for 4h, followed with 48h SF incubation. Cells were then stimulated with 100μM nucleotides for 5min or 20min and western blotting for phospho-Akt and phospho-ERK. Data represented mean ± SEM (n=3) and were analysed by One-way ANOVA and post test was performed with Dunnet's to compare all data sets to the control using Graphpad Prism (**p<0.01, *p<0.05). Blots are representative of 3 independent experiments and data are from 3 independent experiments.

A



B

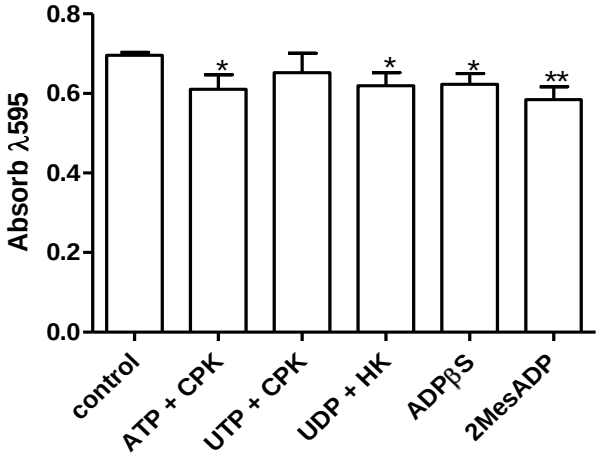


Figure 4.2 Comparison the effect of each nucleotide on the A. regulation of cell cycle and B. cell viability

Hepatocytes were incubated in WME supplemented with ITS and 10% FCS for 4h after plating cell, subsequently cells were changed to SF WME and incubated for 24h. 100μM nucleotides were added and left for 24h as indicated. (A) DNA synthesis was measured by [^{3}H]-thymidine assay and (B) cell viability was measured by MTT assay. Data represented mean ± SEM (n=16) and were analysed by One-way ANOVA and post test was performed with Dunnet's to compare all data sets to the control using Graphpad Prism (**p<0.01, *p<0.05). Data are from 4 representatively independent experiments.

4.2.2 UTP stimulation of primary rat hepatocytes

4.2.2.1 UTP stimulation of phosphorylation of Akt and ERK

UTP induced an increase in the level of Akt and ERK phosphorylation has been shown in Figure 4.1. In the following experiments, UTP concentration-response curve and time course of the phosphorylation of Akt and ERK were investigated to establish the dose-response, extent and duration of the response to UTP.

Figure 4.3A shows a relatively rapid stimulation of either ERK or Akt phosphorylation by UTP. After exposure to UTP for 1min, both the phosphorylation of Akt and ERK was detected. The level of UTP-induced the phosphorylation of Akt and ERK peaked at 5min after which the stimulation of both decreased. The stimulation of Akt phosphorylation response lasted for about 1h before returning to the control level. The induction of the phosphorylation of ERK was still detectable at 1h, although it was not significantly higher than control level.

Figure 4.3B shows the dose-response curve of UTP stimulation of Akt and ERK phosphorylation. UTP-induced Akt and ERK phosphorylation was evident at 30μM and the response peaked at 100μM (Figure 4.3). The level of the phosphorylation of ERK and Akt increased as the concentration of UTP increased. There was no significant difference in the response to 100μM and 300μM UTP, the two highest concentrations used.

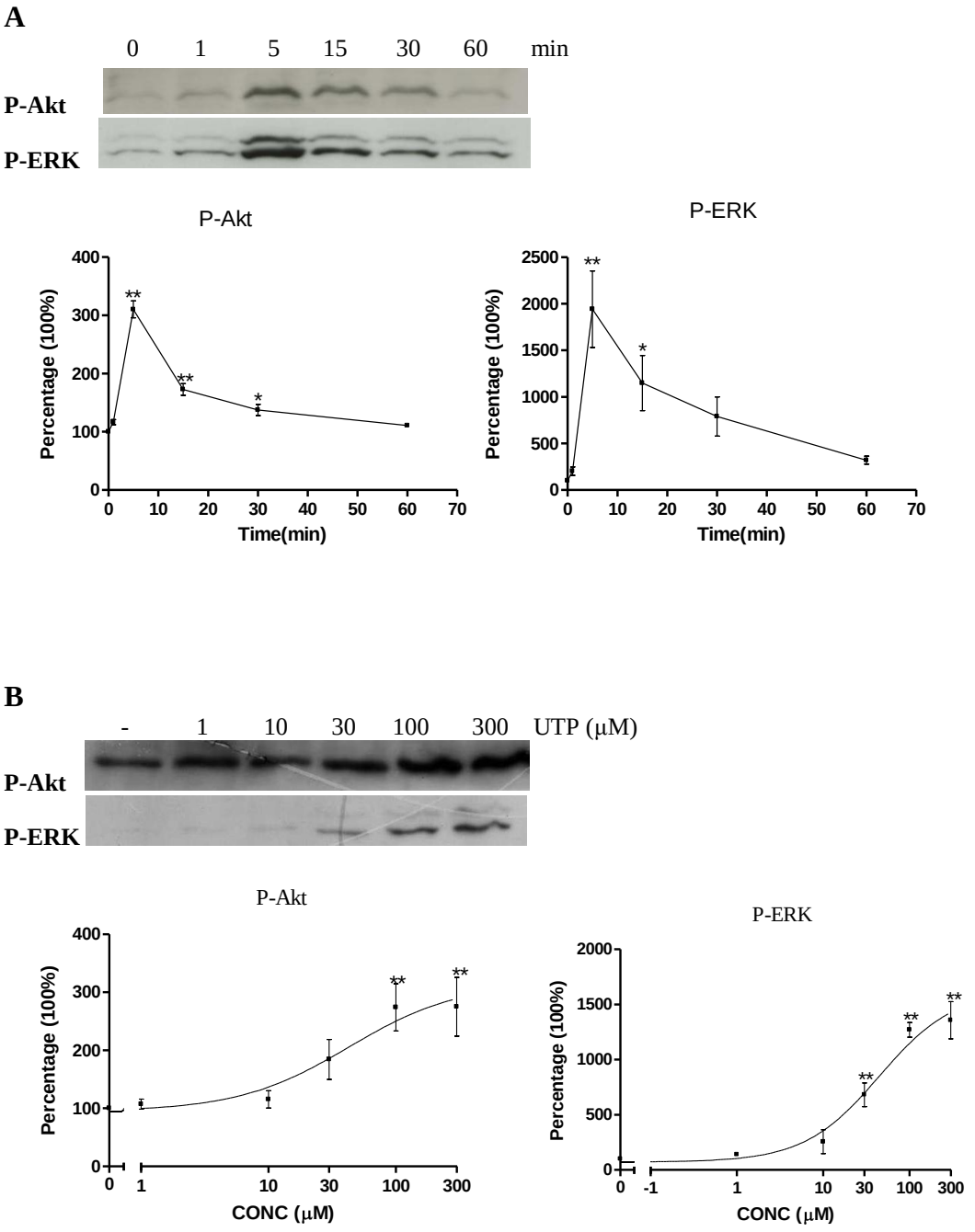


Figure 4.3 A. UTP time course and B. Different concentrations of UTP stimulation of the phosphorylation of Akt and ERK

Hepatocytes were incubated in WME supplemented with ITS and 10% FCS for 4h after plating followed with 48h SF incubation before stimulation. Cells were treated with A. different concentrations of UTP for 5min or B exposed to 100μM UTP for different durations. Phospho-Akt and phospho-ERK response were measured by western blotting. Data represented mean ± SEM (n=3) and were analysed by One-way ANOVA and post test was performed with Dunnet's to compare all data sets to the control using Graphpad Prism (**p<0.01, *p<0.05). Blots are representative of 3 independent experiments and data are from 3 independent experiments.

4.2.2.2 UTP stimulation of hepatocyte DNA synthesis

The effect of different concentrations of UTP on DNA synthesis was determined by measuring [^{3}H]-thymidine incorporation. Hepatocytes were exposed to different concentrations of UTP for 24h before [^{3}H]-thymidine assay.

On exposure of cells to UTP for 24h, every concentration tested failed to induce the hepatocyte cell cycle progression, as shown in Figure 4.4. Furthermore, 300μM UTP inhibited [^{3}H]-thymidine incorporation into DNA, although this concentration of UTP gave the most potent response of induction of Akt and ERK phosphorylation. This could be because the high concentration of UTP induced cell apoptosis (Coutinho-Silva *et al*., 2005). Therefore, 100μM of UTP was chosen to use in further experiment, since this concentration gave a strong stimulation of phospho-Akt and phospho-ERK in hepatocyte and did not cause the inhibition of DNA synthesis.

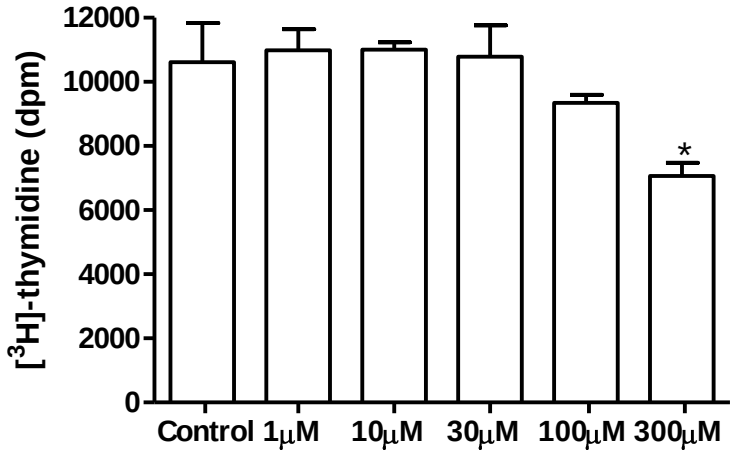


Figure 4.4 UTP concentration-response curve of stimulation hepatocyte DNA synthesis

Hepatocytes were incubated in WME supplemented with ITS and 10% FCS for 4h after plating cell, subsequently cells were changed to SF WME and incubated for 24h. Different concentrations of EGF was added to the cells and left for 24h. (A) DNA synthesis was measured by [^{3}H]-thymidine assay and (B) cell viability was measured by MTT assay. Data represented mean ± SEM (n=8) and were analysed by One-way ANOVA and post test was performed with Dunnet's to compare all data sets to the control using Graphpad Prism (*$p<0.05$). Data are from two independent experiments.

4.3 Discussion

ATP and UTP produced the largest response of induction of Akt and ERK phosphorylation which suggested that $P2Y_2$ and $P2Y_4$, the receptors for these two agonists (Dixon *et al*., 2000), were the main receptors inducing the phosphorylation of Akt and ERK. Although the function of the $P2Y_{13}$ is still not clear, the slight stimulation of the phosphorylation of Akt and ERK caused by ADPβS (Figure 4.1) indicated that $P2Y_{13}$ may be also involved in the nucleotides inducing the phosphorylation of Akt and ERK in rat hepatocytes. 2MesADP, which is the agonist of $P2Y_1$ (Dixon *et al*., 2000) and $P2Y_{13}$ (Marteau *et al*., 2003), also produced a slight stimulation of ERK phosphorylation. UDP, which stimulates $P2Y_6$ (Chang *et al*., 1995), did not stimulate Akt and ERK phosphorylation, suggesting that $P2Y_6$ may be not involved.

$P2Y_2$ regulates cell cycle progression and proliferation, while $P2Y_4$ is mainly involved in the regulation of epithelial response have been demonstrated by several groups (Greig *et al*., 2003; Robaye *et al*., 2003; Schafer *et al*., 2003). The phosphorylation of MAPK including ERK1/2, JNK and p38 following activation of the $P2Y_2$ has been reported by many groups (Soltoff, 1998; Soltoff *et al*., 1998; Gendron *et al*., 2003). This resulted in cell proliferation and/or migration in many cell types such as human epidermal keratinocytes, lung epithelial tumour cells, glioma cells and smooth muscle cells (Wilden *et al*., 1998; Tu *et al*., 2000; Seye *et al*., 2002; Greig *et al*., 2003; Schafer *et al*., 2003). Furthermore, ATP/UTP induced HeLa cell proliferation via activation of the $P2Y_2$ receptor through the regulation of PI3K/Akt and MEK/ERK signalling pathways (Muscella *et al*., 2003). All these data suggested that the $P2Y_2$ receptor plays

an essential role in nucleotides-induced proliferation-related response. On the other hand, $P2Y_4$ is mainly thought to involve in the regulation of the epithelial response. For example, UTP mediated epithelial secretion of chloride in the colon was reported via the activation of the $P2Y_4$ (Ghanem *et al*., 2005). In the liver, $P2Y_4$ has also been proved to play a major role in the mediation of ion secretion with the addition of other P2Y subtypes such as $P2Y_1$, $P2Y_2$ and $P2Y_6$ (Robaye *et al*., 2003). The use of the $P2Y_4$ receptor knockout mice model provided strong evidence that $P2Y_4$ is not involved in the regulation cell proliferation. The result showed that $P2Y_4$-null mice have apparently normal behaviour, growth and reproduction. This indicated that $P2Y_4$ may be not involved in the regulation of hepatocyte proliferation and/or DNA synthesis. Therefore, in hepatocytes, it was possible that the $P2Y_2$ receptor is the major receptor, which regulates the proliferation-related signals such as Akt and ERK.

Figure 4.1 also shows that ATP produced a greater stimulation of Akt and ERK phosphorylation than UTP. This could be due to various reasons. For example, ATP resulted in a greater stimulation of the activation of $P2Y_2$ and/or $P2Y_4$ receptor than UTP. However, in the experiment, ATP and UTP were used at 100μM, which has been demonstrated as a supra-optimal concentration inducing the strongest response in human hepatocytes (Schofl *et al*., 1999). Therefore, the stronger Akt and ERK phosphorylation stimulated by ATP might not be because ATP induced stronger activation of $P2Y_2$ and/or $P2Y_4$ receptors. Another possibility was that ATP activated other P2Y subtypes, which may stimulate the phosphorylation of Akt and ERK in rat hepatocytes. The results show that $P2Y_{13}$ and/or $P2Y_1$ (ADPβS, 2MesADP) produced a slight stimulation of the phosphorylation of Akt and ERK (Figure 4.1). ATP is a weak

partial agonist at $P2Y_1$ and $P2Y_{13}$, the ability of ATP to activate $P2Y_1$ and $P2Y_{13}$ may depend on the level of expression of the receptor, has been reported (Marteau *et al*., 2003; Dixon *et al*., 2000). Therefore, ATP might stimulate the phosphorylation of Akt and ERK through the activation of $P2Y_1$ and/or $P2Y_{13}$ and hence produce a stronger stimulation response. Moreover, although ATP gave a small increase in DNA synthesis (Figure 4.2A) in hepatocytes, it also reduced cell viability (Figure 4.2B). This might be because ATP induced DNA synthesis in some cells, however, it also arrested most cells at S and/or G1 phase and may induce apoptosis after that. It indicated the multiple functions of ATP and makes it difficult to understand. In order to avoid the interference of $P2Y_1$ and/or $P2Y_{13}$ activation and the multiple biological effects may be resulted by ATP; UTP was choosed to further study the possible role for $P2Y_2$ in hepatocyte proliferation.

The results presented here show that, in primary rat hepatocytes, UTP did not induce DNA synthesis and/or proliferation; although it caused the phosphorylation of both Akt and ERK, which have been shown to increase cell survival and control cell proliferation (Woodgett, 2005; Charmbard *et al*., 2007). Moreover it has been recently reported that, in human endometrial stromal cells, activation of the $P2Y_2$ receptor leaded to the inhibition of cell viability (Chang *et al*., 2008). They reported that ATP activated the PLC/PKC/ERK signalling pathway leading to a reduction of cell viability. Furthermore, the high concentration (100μM) of nucleotides used in our experiments also could be the reason for the decrease of hepatocyte cell viability. For instance, Burnstock and colleagues showed that a high concentration of ATP (>10μM) induced cell apoptosis through $P2X_7$ and maybe $P2Y_2$ in HCT8 and Caco-2 cell lines (Coutinho-Silva *et al*.,

2005). The effect of $P2Y_2$ receptor induction of apoptosis and anti-proliferation have also been found in other cells such as human epithelial carcinoma cells, colorectal and esophageal cancer cells (Hopfner *et al*., 1998; Hopfner *et al*., 2001; Maaser *et al*., 2002). As shown in Figure 4.4, a high concentration of UTP (300μM) decreased the level of [^{3}H]-thymidine incorporation into DNA, which indicated that, at high concentration, nucleotides may have anti-proliferative and/or apoptotic effects in rat hepatocytes. However, the different pathways may cause different effects of $P2Y_2$ activation on cell proliferation and DNA synthesis. For example, the activation of MAPK signalling pathway through the $P2Y_2$ induced src-mediated transactivation of growth factor receptors has been reported to stimulate COS-7 cell proliferation (Luttrell *et al*., 1997). From our results, 100μM UTP had no effect on hepatocyte DNA synthesis and cell viability although UTP clearly stimulated Akt and ERK phosphorylation at this concentration. Therefore, in further experiments, this concentration of UTP was used as a sample, which stimulates the phosphorylation of proliferation-related proteins such as Akt and ERK without inducing cell cycle progression.

Chapter 5

Comparison of Growth Factor and Nucleotide Stimulation in Primary Rat Hepatocytes

5.1 Introduction

Previous results have shown that EGF, a growth factor (Chapter 3) or UTP, a nucleotide (Chapter 4), can stimulate the phosphorylation of Akt and ERK. Both Akt and ERK cascades have been shown to play a central role in the regulation of the hepatocyte cell cycle progression. However, UTP did not induce hepatocyte cell cycle progression, while EGF triggered cell entry into S phase. These results suggest that EGF and UTP may stimulate Akt and ERK phosphorylation to a different extent or via different signalling pathways, which then lead to the different biological effects. Therefore, the EGF and UTP stimulation in primary hepatocytes were further studied.

It has been shown that ATP stimulated Muller glial cell mitosis via PI3K/Akt and ERK1/2 pathways by trans-activation of the EGFR (Milenkovic *et al*., 2003). They claimed that ATP activated the P2Y receptor to releasing HB-EGF from the Muller cell matrix, which then activated the EGFR mitogenic signalling. Also, another group reported that the P2Y receptor induced MAPK activation in rat-1 fibroblasts and PC12 cells was dependent on the trans-activation of the EGFR (Soltoff, 1998; Soltoff *et al*., 1998). Furthermore, Schlessinger and colleagues concluded that the src family of tyrosine kinases played an essential role in the GPCR trans-activation of the EGFR (Andreev *et al*., 2001). Therefore, the effects of src and EGFR-mediated tyrosine kinase on UTP and EGF stimulation of hepatocytes were investigated. The activation of src was inhibited using PP2, a potent and selective inhibitor of the src family of tyrosine kinases (Hanke *et al*., 1996), while EGFR-mediated tyrosine kinase activity was blocked using AG1478, which inhibits EGFR homo-dimerisation dependent tyrosine kinase activity (Osherov and Levitzki, 1994).

Previously we have provided strong evidence that the PI3K/Akt pathway is crucial for EGF induction of rat hepatocyte cell cycle progression (Chapter 3). The inhibition of the activation of Akt prevented growth factor inducing cell enter S phase. Also either EGF or UTP induced the phosphorylation of Akt in freshly isolated rat hepatocytes. It has been suggested in certain cells that the activation of Akt may be PI3K independent. For instance, it has been reported that the inhibition of PI3K by wortmannin did not affect dopamine induction of the phosphorylation of Akt at Thr308 in striatal neurons (Brami-Cherrier *et al*., 2002). It also has been shown that the activation of Akt may depend on cyclic AMP protein kinase in HEK-293 cells (Filippa *et al*., 1999). Moreover, in NG108 neuroblastoma cells, N-methyl-D-aspartate (NMDA) activated Akt directly via Ca^{2+}/calmodulin-dependent protein kinase kinase (CaM-KK) (Yano *et al*., 1998). Therefore, it is possible that the different effects of EGF and UTP on the rat hepatocyte cell cycle may be due to EGF and UTP inducing Akt phosphorylation through different signalling pathways. Therefore, the activation of PI3K and Akt were blocked by LY294002 and A443654 respectively, EGF and UTP stimulation of Akt and GSK-3 phosphorylation were then investigated.

Traditionally, ligand binding to RTKs or GPCRs activates signal transduction and induces receptor internalisation, which is an effective mechanism to terminate receptor activation. However, more recent studies demonstrated that receptors may continue signal from endosomes. In fact, certain signalling events such as ERK phosphorylation appeared to require receptor internalisation (reviewed by (McPherson *et al*., 2001b; Tobin *et al*., 2008). Therefore, whether the different of EGF and UTP effects were due to these two receptors signal from different intracellular locations was investigated.

Con A, a non-selective endocytosis inhibitor (Xiang *et al*., 2002), was used to inhibit ligand induced EGFR and $P2Y_2$ receptor internalisation.

5.2 Results

5.2.1 Comparison of EGF and UTP stimulation time course

In order to investigate whether EGF induced hepatocyte cell cycle progression was due to a more prolonged EGF stimulation of the PI3K/Akt and MEK/ERK signalling pathways than UTP, the time courses of EGF and UTP induction of Akt, ERK and GSK-3 phosphorylation were measured by western blotting.

5.2.1.1 EGF and UTP time course of phospho-Akt

Figure 5.1 shows the extent and duration of EGF and UTP stimulation of Akt phosphorylation. EGF and UTP rapidly stimulated the phosphorylation of Akt and the response rose to peak at approximately 5min. The stimulation gradually decreased and at 1h either EGF or UTP stimulation of the phosphorylation of Akt declined to control levels, which was 1.4 ± 0.3 and 1.3 ± 0.2 fold of unstimulated cells respectively. Furthermore, 3nM EGF and 100μM UTP produced a very similar level of stimulation of Akt phosphorylation, the maximum response being 3.2 ± 0.5 and 4.2 ± 0.7 fold over control respectively.

5.2.1.2 EGF and UTP time course of phospho-GSK-3

UTP increased the level of GSK-3 phosphorylation and peaked at 10min (1.6 ± 0.2 fold), while the maximum EGF stimulation was measured around 10 to 15min (2.5 ± 1 to 2.7 ± 0.7 fold), as shown in Figure 5.2. After that UTP stimulation of GSK-3 phosphorylation decreased, returning to control levels after 40min. However, EGF

stimulation of GSK-3 phosphorylation decreased gradually. At 1h, the level of GSK-3 phosphorylation was still 1.3 ± 0.6 fold higher than unstimulated cells.

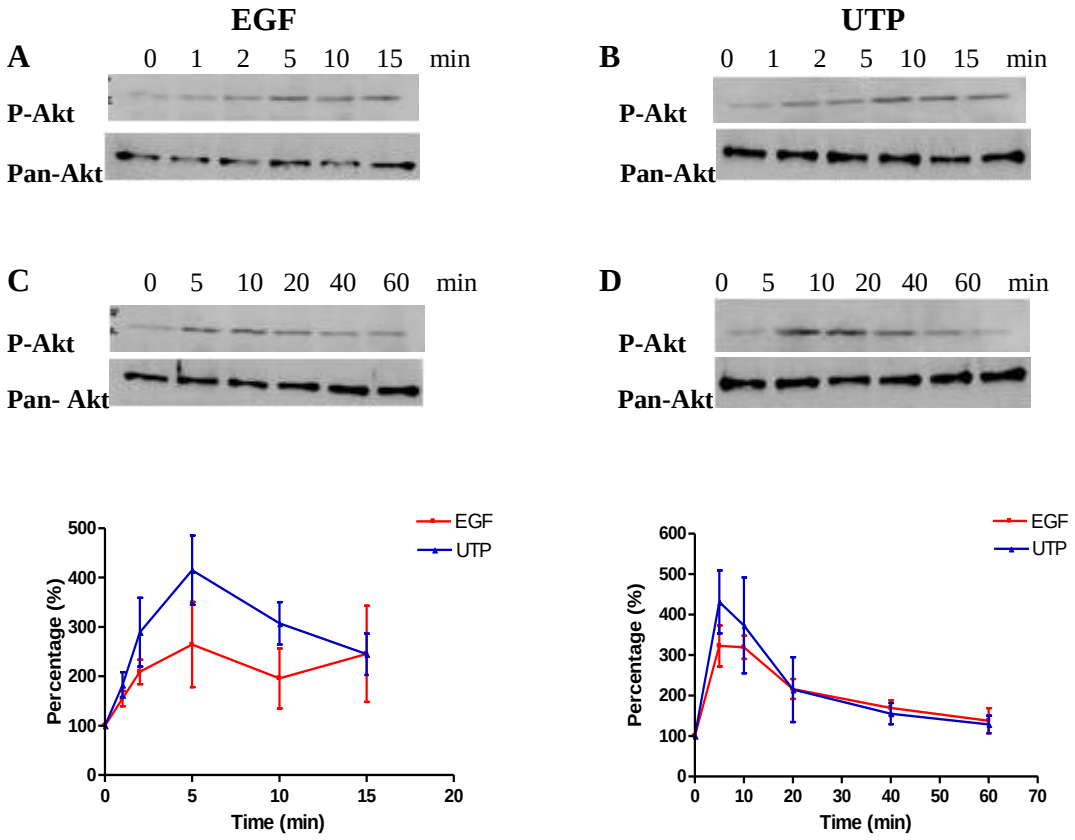


Figure 5.1 EGF stimulation of Akt phosphorylation A. short time course C. long time course and UTP stimulation of Akt phosphorylation B. short time course D. long time course.

Primary rat hepatocytes were cultured in WME supplemented with ITS and 10% FCS after plating for 4h, followed with 48h serum free incubation. Cells were then stimulated with 3nM EGF or 100μM UTP at different time points as indicated and western blotting for phospho-Akt. Data represented mean ± SEM (n=3) and were analysed by two-way ANOVA using Graphpad Prism (not significant different between EGF and UTP stimulation). Western blots are representative of 3 independent experiments and statistic data are from 3 independent experiments.

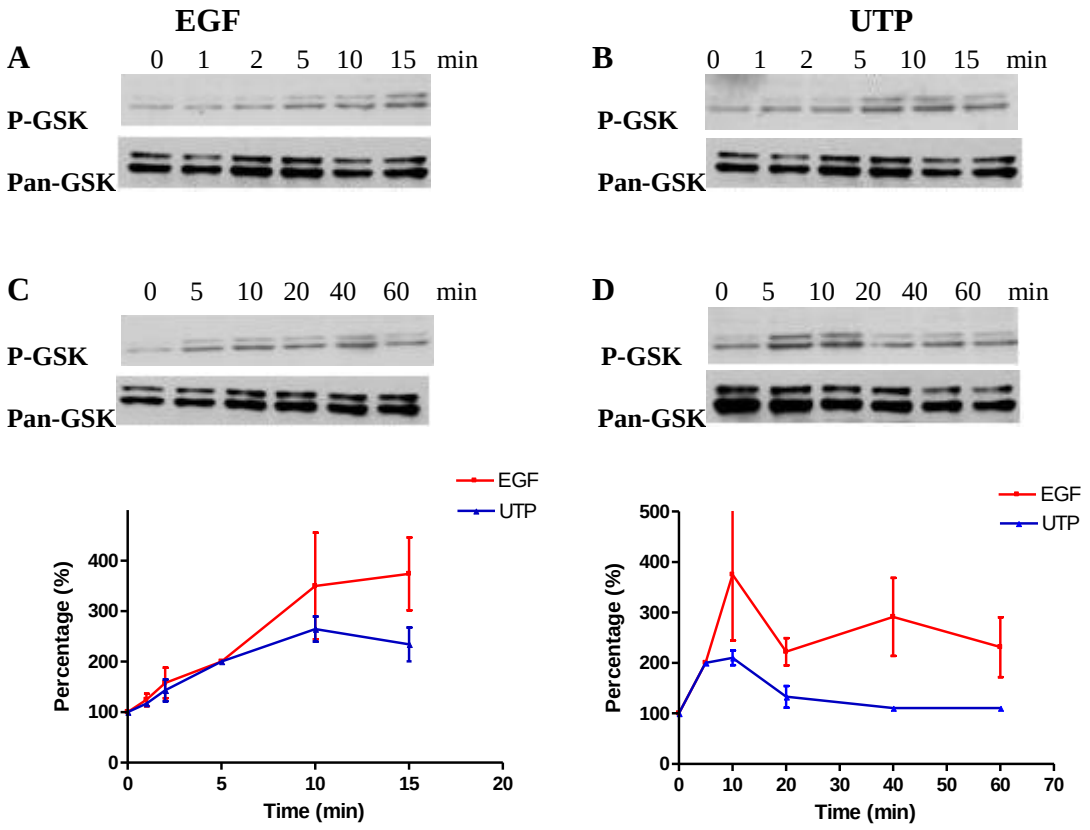


Figure 5.2 EGF stimulation of GSK phosphorylation A. short time course C. long time course and UTP stimulation of Akt phosphorylation B. short time course D. long time course.

Primary rat hepatocytes were cultured in WME supplemented with ITS and 10% FCS after plating for 4h, followed with 48h serum free incubation. Cells were then stimulated with 3nM EGF or 100μM UTP at different time points as indicated and western blotting for phospho-GSK-3. Data represented mean ± SEM (n=3) and were analysed by two-way ANOVA using Graphpad Prism (not significant different between EGF and UTP stimulation). Western blots are representative of 3 independent experiments and statistic data are from 3 independent experiments.

5.2.1.3 EGF and UTP time course of P-ERK

Figure 5.3 shows a significant difference between EGF and UTP stimulation of ERK phosphorylation in hepatocytes. UTP induced a rapid increase of the level of the phosphorylation of ERK. The response was measured after exposure of cells to UTP for 1min and the level of UTP induction of the phosphorylation of ERK peaked at 5min. The stimulation quickly decreased after that, and almost reduced to control level after 1h. However, EGF stimulation of ERK phosphorylation was firstly detected at 5min. Then the response gradually rose and reached the maximum at 1h, which was about 20 fold higher than control levels. It shows a significant difference between the EGF and UTP stimulation of ERK phosphorylation time course.

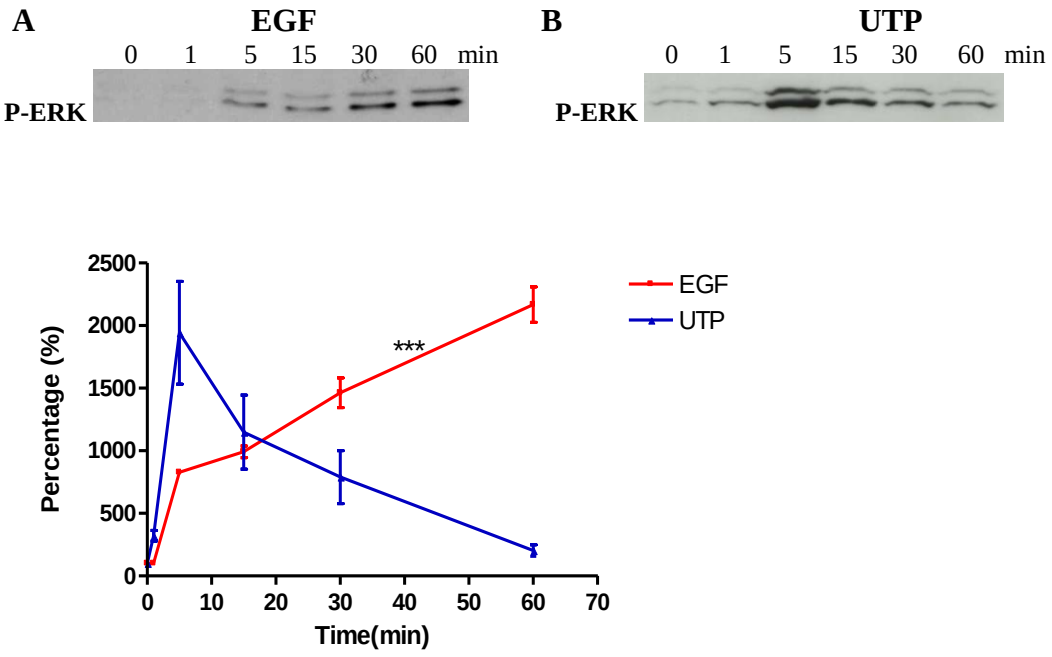


Figure 5.3 A. EGF and B. UTP stimulation of ERK phosphorylation time course

Blot A is from Figure 3.3 and B is from Figure 4.3.

5.2.2 Comparison of EGF and UTP stimulation signalling pathways

5.2.2.1 Cross-talking between the $P2Y_2$ receptor (GPCR) and the EGFR (Growth Factor Receptor)

5.2.2.1.1 Src-mediated cross-talking between the GPCR and growth factor receptor

The src family of tyrosine kinases have been reported as one of the major factors mediating the GPCR trans-activated growth factor receptor (Andreev *et al*., 2001). The possibility of src-dependent cross-talk between the EGFR and the $P2Y_2$ receptor was investigated by treatment with PP2, an inhibitor of the src tyrosine kinase family; EGF and UTP stimulation of Akt and ERK phosphorylation were measured by western blotting.

The results show that the inhibition of the src kinase family activity did not significantly affect EGF or UTP stimulation of Akt or ERK phosphorylation in primary rat hepatocytes (Figure 5.4).

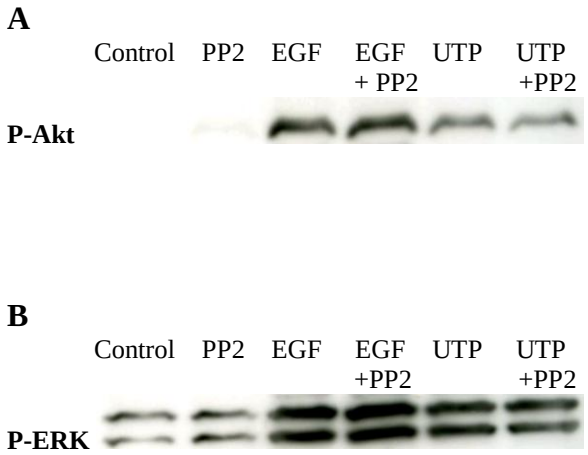


Figure 5.4 Effect of PP2 on EGF and UTP induction of A. Akt phosphorylation and B. ERK phosphorylation

Primary rat hepatocytes were cultured in WME supplemented with ITS and 10% FCS after plating for 4h, followed with 48h serum free incubation. Cells were pre-incubated with 100μM PP2 for 15min and then stimulated with 3nM EGF or 100μM UTP for 5min or 20min. Western blotting of phospho-ERK (20min) and phospho-Akt (5min) were measured. Blots are representative of 3 independent experiments.

5.2.2.1.2 The role of tyrosine kinase in the GPCR trans-activated growth factor receptor

In rat-1 fibroblasts and PC12 cells, the P2Y receptor induced MAPK activation was dependent on cross-talk with the EGFR (Soltoff, 1998; Soltoff *et al*., 1998). This suggested that the EGFR is downstream target of the P2Y receptor, and inhibition of the EGFR tyrosine kinase would block response from both receptors. Here we used AG1478 to inhibit EGFR-activated tyrosine kinase activity, EGF and UTP simulation of the phosphorylation of Akt and ERK and [^{3}H]-thymidine incorporation into DNA were measured.

Figure 5.5 shows that pre-incubation with 300nM AG1478 effectively abolished EGF stimulation of Akt and ERK phosphorylation. However, the inhibition of tyrosine kinase activity had no significantly effect on UTP induction of Akt and ERK phosphorylation in hepatocytes.

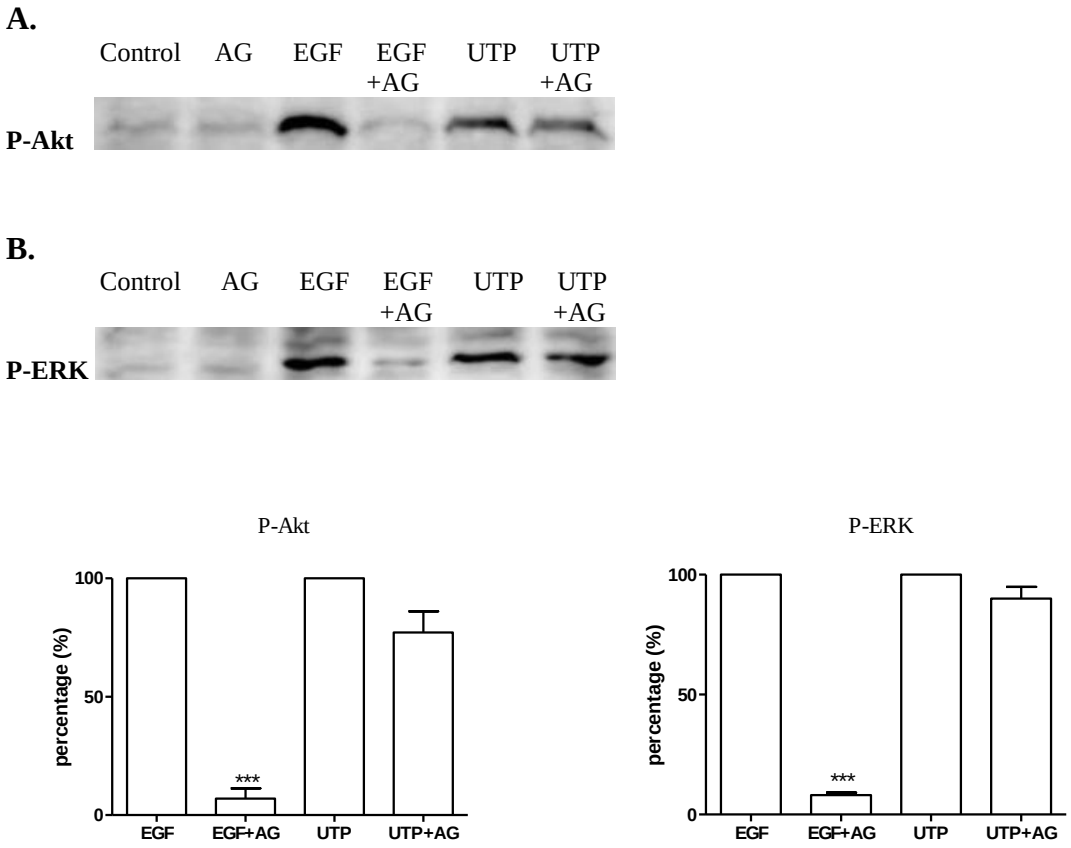


Figure 5.5 Effect of AG1478 on EGF and UTP induction of A. Akt phosphorylation and B. ERK phosphorylation

Primary rat hepatocytes were cultured in WME supplemented with ITS and 10% FCS after plating for 4h, followed with 48h SF incubation. Cells were pre-incubated with 300nM AG1478 for 15min and then stimulated with 3nM EGF or 100μM UTP for 5min or 20min. Western blotting of phospho-ERK (20min) and phospho-Akt (5min) were measured. Data represented mean ± SEM (n=3) and were analysed by One-way ANOVA and post test was measured with Bonferroni's to compare all data sets using Graphpad Prism (***$p<0.001$). Western blots are representative of 3 independent experiments and statistic data are from 3 independent experiments.

5.2.2.2 Comparison of EGF and UTP stimulation of PI3K/Akt signalling pathway

5.2.2.2.1 The role of PI3K on EGF and UTP stimulation signalling pathway

In order to investigate whether EGF and UTP stimulation of Akt phosphorylation is PI3K activity dependent, LY294002 was used to block PI3K activation and EGF and UTP induction of Akt phosphorylation was measured by western blotting. Furthermore, EGF and UTP induction of the phosphorylation of GSK-3, Akt downstream protein, was also measured with LY294002 present.

The inhibition of PI3K activity completely blocked EGF and UTP stimulation of Akt phosphorylation (Figure 5.6A). Furthermore, LY294002 significantly reduced EGF and UTP induction of the phosphorylation of GSK, a downstream protein of Akt, to 21% and 53% respectively.

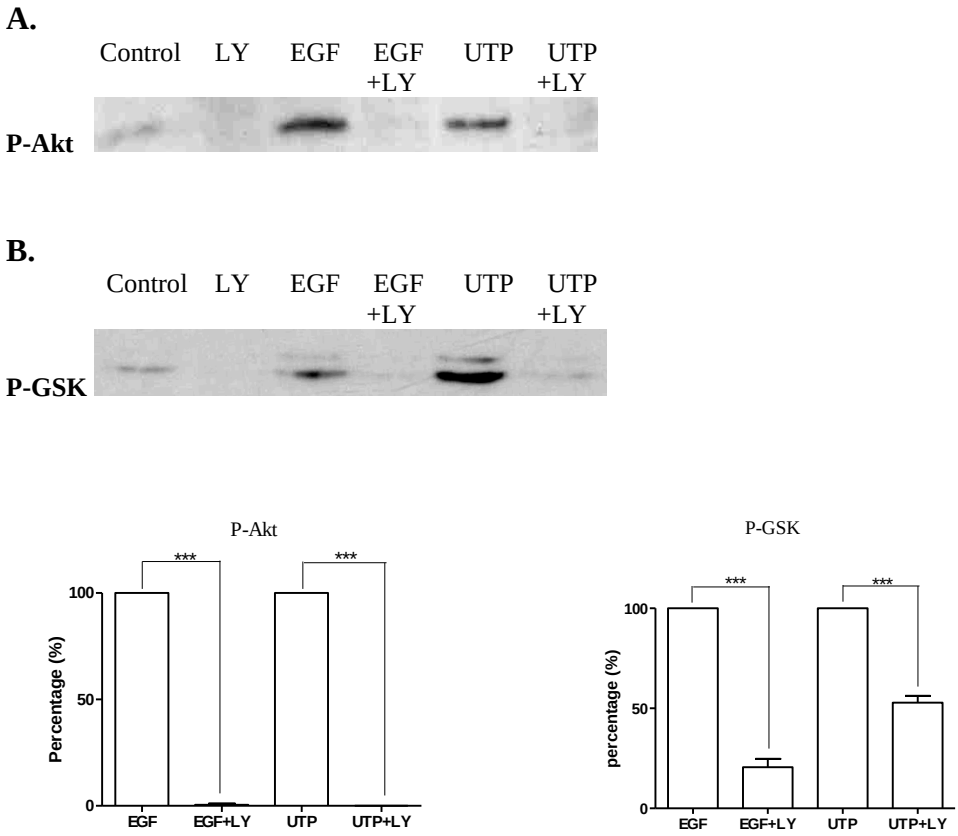


Figure 5.6 Effect of LY294002 on EGF and UTP induction of A. Akt phosphorylation and B. GSK phosphorylation

Primary rat hepatocytes were cultured in WME supplemented with ITS and 10% FCS after plating for 4h, followed with 48h SF incubation. Cells were pre-incubated with 10μM LY294002 for 15min and then stimulated with 3nM EGF or 100μM UTP for 5min. Western blotting of phospho-GSK and phospho-Akt were measured. Data represented mean ± SEM (n=3) and were analysed by One-way ANOVA and post test was measured with Bonferroni's to compare all data sets using Graphpad Prism (***$p<0.001$). Western blots are representative of 3 independent experiments and statistic data are from 3 independent experiments.

5.2.2.2.2 The role of Akt in EGF and UTP stimulation signalling pathway

To detect whether EGF and UTP stimulation of the phosphorylation of GSK-3 was directly through Akt activation, Akt activity was inhibited using A443654.

Figure 5.7 shows that the inhibition of Akt activation completely blocked EGF induction of GSK-3 phosphorylation in rat hepatocytes. A443654 greatly reduced UTP-mediated the phosphorylation of GSK-3 to 6.9% ± 1.5%. However, the level of EGF and UTP stimulation of GSK-3 phosphorylation was significantly different.

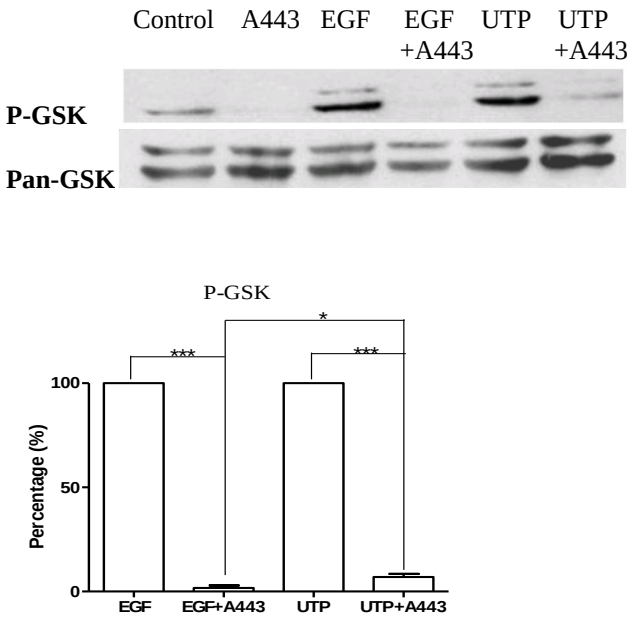


Figure 5.7 Effect of A443654 on EGF and UTP stimulation of GSK phosphorylation

Primary rat hepatocytes were cultured in WME supplemented with ITS and 10% FCS after plating for 4h, followed with 48h SF incubation. Cells were pre-incubated with 3μM A443654 for 30min and then stimulated with 3nM EGF or 100μM UTP for 5min. Western blotting of phospho-GSK were measured. Data represented mean ± SEM (n=3) and were analysed by One-way ANOVA and post test was measured with Bonferroni's to compare all data sets using Graphpad Prism (*$p<0.05$, ***$p<0.001$). Western blots are representative of 3 independent experiments and statistic data are from 3 independent experiments.

5.2.2.3 Effect of the internalisation on EGF and UTP stimulation

5.2.2.3.1 Effect of concanovalin A (con A) on EGF and UTP stimulation

The role of internalisation of EGF/EGFR and UTP/$P2Y_2$ was studied using con A, a non-selective internalisation inhibitor. EGF and UTP stimulation of Akt and ERK phosphorylation were measured.

As shown in Figure 5.8, the inhibition of ligand/receptor internalisation in primary rat hepatocytes with con A strongly reduced EGF induction of the increase of Akt and ERK phosphorylation. However, con A did not inhibit UTP stimulation in hepatocytes. In fact, the inhibition of internalisation significantly increased UTP stimulation of Akt and ERK phosphorylation to 153% ± 35% and 170% ± 36% respectively in hepatocytes.

The effect of con A on UTP stimulation of Akt and ERK phosphorylation may be a result of prolonged UTP stimulation at the cell surface, which would maintain the receptor on a longer period of time. Also, it could be due to con A increased the size of the response without changing the time course. In order to distinguish between these possibilities, a time course of UTP stimulation was measured, while cells were treated with/without con A.

Figure 5.9 shows that the con A-mediated inhibition of UTP/$P2Y_2$ did not affect the duration of UTP stimulation of Akt and ERK phosphorylation in hepatocytes. However, blocking internalisation increased the extent of UTP stimulation. As shown in Figure 5.9A, UTP stimulation of Akt phosphorylation was significantly increased in con A treated cells. The data (Figure 5.9B) indicates that UTP induced ERK

phosphorylation was also increased by con A, although the effect was not statistically significant.

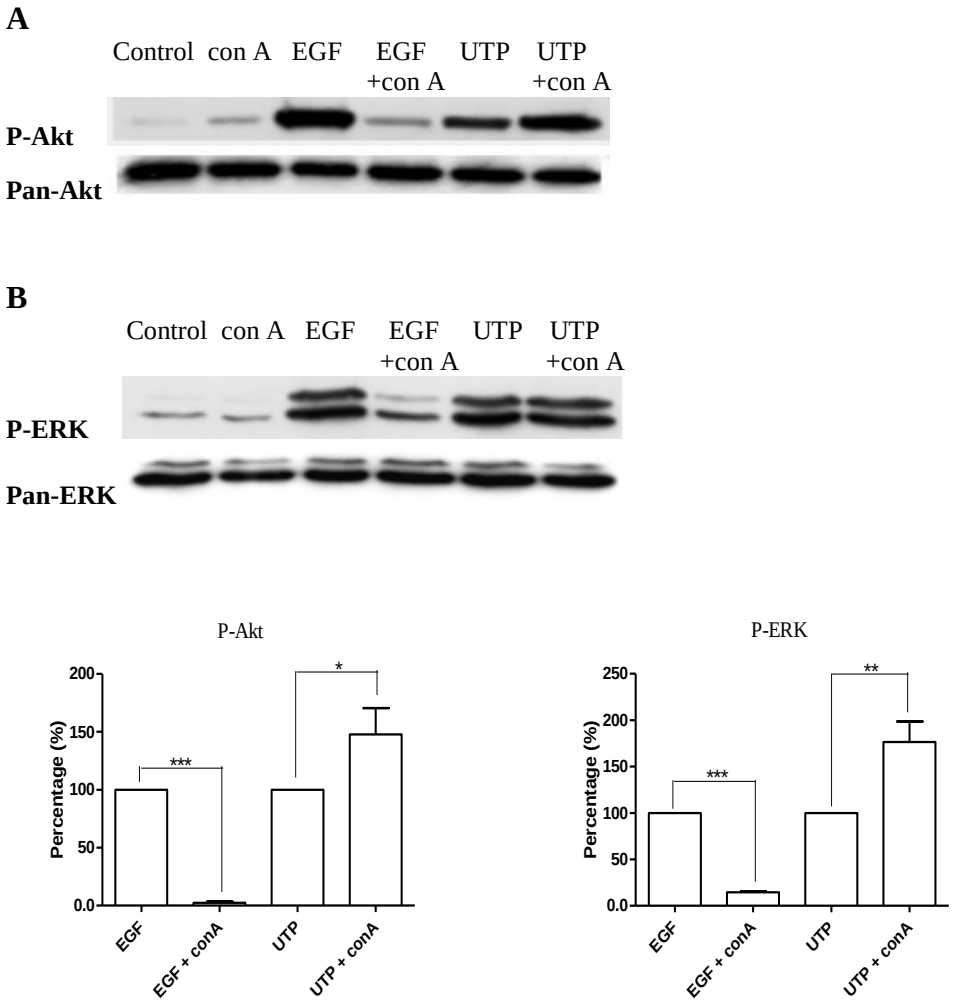


Figure 5.8 Effect of con A on EGF and UTP stimulation of A. Akt and B. ERK phosphorylation

Primary rat hepatocytes were cultured in WME supplemented with ITS and 10% FCS after plating for 4h, followed with 48h SF incubation. Cells were pre-incubated with 250μg/ml con A for 15min and then stimulated with 3nM EGF or 100μM UTP for 5min and 20min. Western blotting of phospho-ERK/Pan-ERK (20min) and phospho-Akt/Pan-Akt (5min) were measured. Data represented mean ± SEM (n=3) and were analysed by One-way ANOVA and post test was measured with Bonferroni's to compare selected pairs of columns using Graphpad Prism (*p<0.05, **p<0.01, ***p<0.001). Western blots are representative of 3 independent experiments and statistic data are from 3 independent experiments.

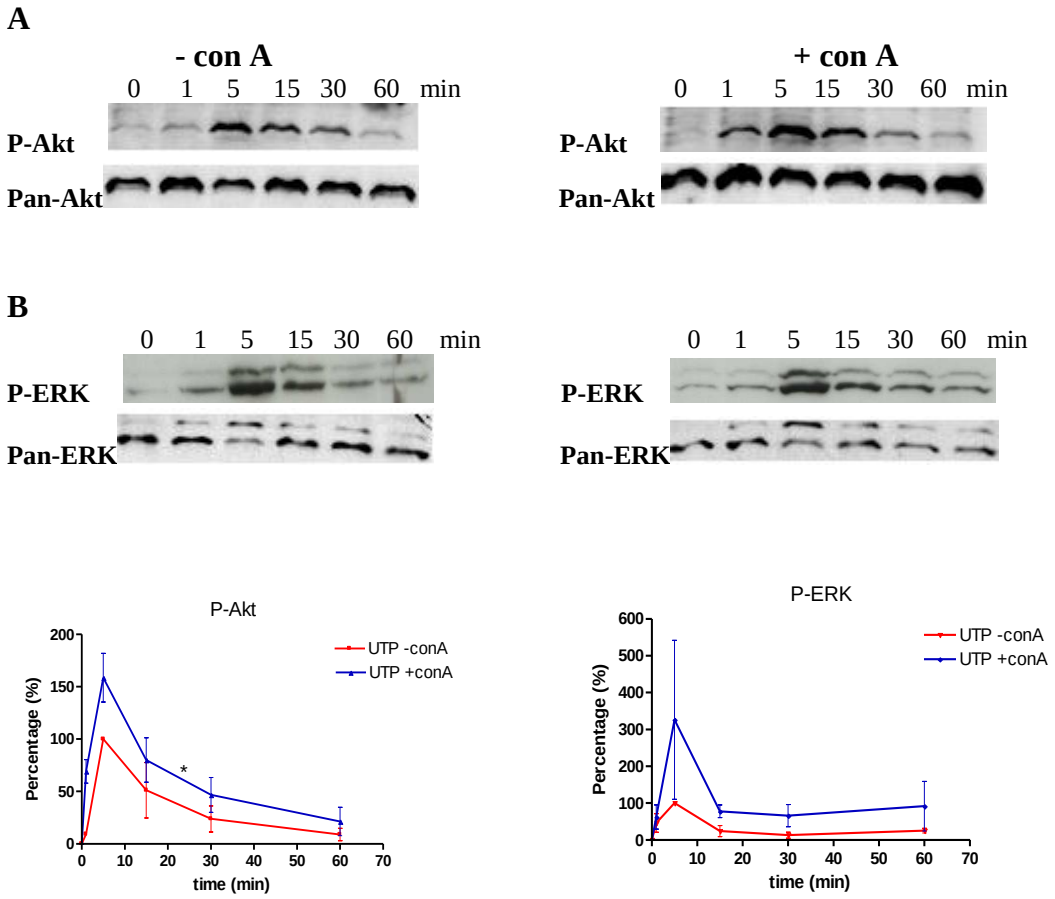


Figure 5.9 Effect of con A on UTP stimulation of A. Akt and B. ERK phosphorylation at different time points

Primary rat hepatocytes were cultured in WME supplemented with ITS and 10% FCS after plating for 4h, followed with 48h SF incubation. Cells were pre-incubated with 250µg/ml con A for 15min and then stimulated with 100µM UTP as indicated. Western blotting of phospho-ERK/Pan-ERK and phospho-Akt/Pan-Akt were measured. Data represented mean ± SEM (n=3) and were analysed by Two-way ANOVA (related values are spread across a row) (*P<0.05). Western blots are representative of 3 independent experiments and statistic data are from 3 independent experiments.

5.3 Discussion

Nucleotides such as ATP could induce cell cycle progression and cell proliferation via PI3K/Akt and ERK1/2 pathways (Milenkovic *et al*., 2003). Furthermore, in many cells, nucleotide/P2Y receptor-induced Akt and ERK phosphorylation were mediated through the trans-activation of growth factor receptors such as the EGFR (Soltoff, 1998; Soltoff *et al*., 1998; Milenkovic *et al*., 2003). However, in primary hepatocytes, the results show that UTP/$P2Y_2$ (GPCR) stimulation of the phosphorylation of Akt and ERK did not depend on the trans-activation of EGFR (growth factor receptor). Either inhibition of the src family tyrosine kinase activity, which plays an essential role in the GPCR tranactivation of the EGFR (Andreev *et al*., 2001), or directly block of the EGFR tyrosine kinase activity did not affect the UTP/$P2Y_2$ stimulation.

From other works, Akt could be induced via either PI3K dependent or independent signalling pathways (Yano *et al*., 1998; Filippa *et al*., 1999; Brami-Cherrier *et al*., 2002; Woodgett, 2005). The inhibition of PI3K activity by LY294002 abolished EGF or UTP induction of the phosphorylation of Akt (Figure 5.6A) suggesting that the activation of Akt is PI3K dependent in hepatocytes. This indicated that the different effect of EGF and UTP on stimulation of hepatocyte cell cycle progression may be not due to EGF and UTP inducing Akt activation via different signalling pathways. However, the inhibition of PI3K-dependent Akt activity did not completely block EGF or UTP stimulation of the phosphorylation of GSK-3, a down-stream regulation target of Akt (Figure 5.6B). Compared to control cells, EGF and UTP stimulation of GSK-3 phosphorylation was reduced to about 20% and 50% respectively in LY294002 pre-treated cells. This suggested that EGF and UTP may stimulate GSK-3 phosphorylation

in an Akt-independent pathway. However, in all experiments, the phospho-Akt was detected using the antibody specific for the Ser473 phosphorylation site. Recent evidence showed that when Akt was phosphorylated at Thr308, it was partially activated; additional phosphorylation at Ser473 fully activated the receptor. Furthermore, the phosphorylation of Akt at Thr308 alone was able to stimulate Akt down-stream regulated proteins such as GSK-3 (Jacinto *et al*., 2006). Therefore, EGF and UTP stimulation of GSK-3 phosphorlyation could still be Akt-dependent, which means the phosphorylation of Akt at Thr308 alone contributed a part of the stimulation of GSK-3 phosphorylation, while phosphorylated Akt at both Ser473 and Thr408 induced full response. It indicated that PI3K activation is necessary for the phosphorylation on Ser473 but may be not on Thr308. Therefore, although exposure of the cells to LY294002 abolished EGF or UTP stimulation of Akt phosphorylation at Ser473 site, it did not completely inhibit the EGF or UTP inducing Akt phosphorylation at Thr308 site and in turn stimulated the phosphorylation of GSK-3. Also it could be because 10μM LY294002 inhibited PI3K-mediated Akt phosphorylation on Ser473 but not Thr308, and higher concentration is required to abolish the phosphorylation on Thr308. Furthermore, when Akt activation was directly inhibited by A443654, EGF-induced Akt phosphorylation was abolished, while UTP-induced response decreased to approximately 10% of the level in control cells (no A443654 present) (Figure 5.7). Therefore, it suggested that EGF stimulation of GSK-3 phosphorylation was Akt-dependent, while UTP stimulation was mainly Akt-regulated.

Differences in the duration and extent of agonist induction of the phosphorylation of one target can result in different biological effects. For instance, in CCL39 cells, α-

thrombin induced a biphasic phosphorylation of ERK, which has a 30min to 1h first stimulation phase and another late phase lasting for several hours. The inhibition of the late phase alone completely abolished α-thrombin stimulated cell cycle progression, while only inhibiting the first phase did not affect the ligand-induced cell cycle progression (Meloche *et al*., 1992). This suggests that one agonist stimulation of the same protein activity at different time or different duration may result in different biological responses. In primary rat hepatocytes, EGF and UTP produced a very similar stimulation of Akt phosphorylation. The response reached a maximum at around 5min and then declined to control level after 1h. GSK-3, which is an Akt downstream protein (Brazil *et al*., 2004), was phosphorylated by either EGF or UTP. UTP and EGF stimulation of GSK-3 phosphorylation peaked at about 10min, which was approximately 5min after the maximum response of EGF and UTP inducing Akt phosphorylation. After that UTP-induced response rapidly decreased to control level, with exposure of cells to UTP for 40min the response was similar to unstimulated cells. EGF stimulation gradually dropped, at 1h EGF stimulation of the increase of the level of GSK-3 phosphorylation was still 1.3 ± 0.6 higher control (unstimulated cells) but not significantly different. However, EGF and UTP induction of ERK phosphorylation was very different in hepatocytes. The time course of UTP stimulation of the phosphorylation of ERK was very similar to the time course of Akt phosphorylation. The response peaked at about 5min and quickly decreased to control level within 1h. EGF stimulation of the phosphorylation of ERK rose much slower than UTP stimulation. The response gradually increased and peaked after exposure of cells to EGF for 1h. There are a lot of evidences that the MEK/ERK signalling cascade is one of the key signalling pathways in response to extracellular signals, which leads to cell

cycle progression and cell proliferation. ERK is a central and key player in this signalling cascade and the activation of ERK is indispensable for cell proliferation in mature differentiated eukaryotic cells (reviewed by (Charmbard *et al*., 2007). Therefore, the more prolonged EGF stimulation of MEK/ERK signalling pathways than UTP may cause the difference between the EGF and UTP stimulation of hepatocyte cell cycle progression.

Figure 5.8 suggests that in primary rat hepatocytes, the internalisation of EGF/EGFR was necessary for EGF stimulation of Akt and ERK phosphorylation, while the internalisation of UTP/P2Y receptor leaded to the reduction of the stimulation. This finding corresponded to Bergeron and colleagues early work, which showed that internalised EGFR maintained phosphorylation of its tyrosine kinase domain and still was activated (*in vivo*) (Lai *et al*., 1989). Furthermore, the same group claimed that in liver the EGF receptor and its down-stream regulated proteins such as Shc, Grb2 and Sos were found in early endosomes, but not in plasma membrane which indicates that EGFR signalling stimulating from intracellular compartment. In other cells, for example BT20, a breast cancer cell line (*in vitro*), the internalised EGFR could activate further signalling cascades such as Akt and ERK from early endosomes (Wang *et al*., 2002). However, in the experiment, the internalisation was inhibited by con A, which has been shown that inhibits EGFR activation by inducing proteolytic cleavage of the EGFR at carboxyl terminus (Tang *et al*., 2000). Therefore, the inhibition of EGF stimulation of Akt and ERK phosphorylation by con A might be due to the con A-mediated inhibition of EGFR internalisation or proteolysis of the receptor. In primary rat hepatocyte, the level of UTP stimulation of ERK and Akt phosphorylation was

increased, when cells were exposed to con A (Figure 5.9). This suggested that UTP/P2Y receptor stimulated signalling on the cell surface and that internalisation of the receptor leaded to the reduction of the stimulation. This was in agreement with the classical model: after binding to an extracellular agonist, the receptor recruits and activates secondary messengers on cell membrane, and the stimulation is then terminated by the internalisation of receptor into lysosomes where the receptor is degradated following ubiquitination. However, recent evidences showed that GPCRs may continue stimulating signalling in endosomes before the degradation. For instance, isoprotereno-induced β2-adrenergic receptor (β2AR) phosphorylation and association with β-arrestins resulted in receptor internalisation and stimulation of ERK and JNK activation (Luttrell *et al*., 1999; McDonald *et al*., 2000). However, inhibition of receptor internalisation increased the size of UTP stimulation of Akt and ERK phosphorylation but did not prolong the time course. This suggested that inhibition of the receptor internalisation into lysosomes, where the receptor is then degradated, did not affect the termination of the receptor activity. It indicated that there may be another factor involved in the regulation of the receptor activation or the receptor can be desensitivated on the cell surface.

Chapter 6

The Effect of Internalisation on UTP/P2Y and EGF/EGFR Stimulation in Primary Rat Hepatocytes

6.1 Introduction

A possible interpretation of previous results is that EGF stimulation might be regulated by the receptor internalisation, while UTP stimulation might be terminated by the internalisation in primary rat hepatocyte (Chapter 5). In the early 1980s, Hubbard and colleagues demonstrated that the the EGFR binding to its ligand (EGF) resulted in the internalisation of the receptor in rat hepatocyte (Dunn and Hubbard, 1984; Dunn *et al*., 1986). Furthermore, internalised EGFR maintained auto-phosphorylation of its tyrosine kinase domain in both endosomes isolated from rat liver homogenates (*in vivo*) and in BT20, a breast cancer cell line (*in vitro*) (Lai *et al*., 1989; Wang *et al*., 2002). However, con A, which was used to inhibit receptor internalisation in previous experiments, also induces EGFR proteolysis in vascular smooth muscle cells and in turn inhibits the signalling (Tang *et al*., 2000). It is not known whether this occurs in other cell types. Therefore, inhibition of EGF stimulation by con A may be due to either the EGFR breakdown or inhibition of the receptor internalisation. In order to further study the role of internalisation in EGF/EGFR and UTP/P2Y receptor signalling, specific inhibitors were used to block ligand-mediated receptor internalisation. Activated receptor can be internalised via several pathways in which clathrin-dependent and caveolin-dependent endocytosis are the best studied. Therefore, the role of these two endocytotic pathways in EGF and UTP stimulation of ERK and Akt phosphorylation were measured. Caveolin-dependent internalisation was inhibited by nystatin, which is a specific caveolin-regulated internalisation inhibitor (Schnitzer *et al*., 1994; Okamoto *et al*., 2000), and clathrin-dependent internalisation was inhibited by sucrose, which inhibits of clathrin-mediated endocytosis (Hansen *et al*., 1993). Furthermore, since it has been demonstrated that dynamin is essential for the formation

and activation of clathrin-coated pits (Schmid, 1997; McNiven, 1998), the clathrin-dependent internalisation was also blocked by transfection of hepatocytes to express Dn-dynamin.

EGFR (also known as ErbB1) appears to signal from endosomes during most of its activated time whereas other members of this family (ErbB2, ErbB3 and ErbB4) prefer to signal from the plasma membrane after EGF induction. For instance, in A431 cells, EGF binding results in the rapidly internalisation of the EGFR into endosomes, and it remains active until degradation. Moreover, approximate 30% of the receptor is recycled to the membrane (Sorkin *et al*., 1991; Wiley, 2003). Therefore, the location of receptor and ligand after stimulation was investigated. For example, EGF was visualised using Alexa-488 conjugated EGF and the EGFR was visualised by application of EGFR antibody, which was then labelled by a cy5 conjugated secondary antibody. The image of the location of EGF and EGFR was then captured at different time points after stimulation.

Recently, Wang and colleagues showed that the internalisation of EGFR is not dependent on its activation, which means that even with inhibition of the EGFR activation, EGF can still result in the receptor internalisation (Wang *et al*., 2002; Wang *et al*., 2005). This finding provides a model to study the possibility of signalling exclusively from internalised EGFR. Therefore, in experiment here, EGF stimulation was inhibited by AG1478, followed by removal of both inhibitor and cell surface binding EGF at which point signalling can only come from internalised receptor. The

effect of internalised EGFR on signalling cascades and cell cycle progression were then investigated.

6.2 Results

6.2.1 The internalisation pathways

6.2.1.1 Effect of the Caveolin-dependent internalisation on the EGF and UTP stimulation

The previous result (Figure 5.8) suggested the importance of receptor internalisation to EGF and UTP stimulation. Here, we further investigated the detail of the EGF/EGFR and UTP/$P2Y_2$ receptor internalisation after stimulation. Firstly we investigated whether the internalisation of the EGFR and $P2Y_2$ receptor are caveolin-dependent using nystatin, a specific caveolin-mediated internalisation inhibitor.

As shown in Figure 6.1, nystatin did not have a significant effect on EGF stimulation of Akt and ERK phosphorylation, which suggested that the EGF/EGFR internalisation is caveolin-independent. However, nystatin increased the UTP stimulation of Akt and ERK phosphorylation to 1.2 ± 0.1 and 2.0 ± 0.2 fold over control levels (only UTP stimulation), respectively, which indicated that caveolin-mediated receptor internalisation played an important role in the regulation of $P2Y_2$ receptor degradation in hepatocytes.

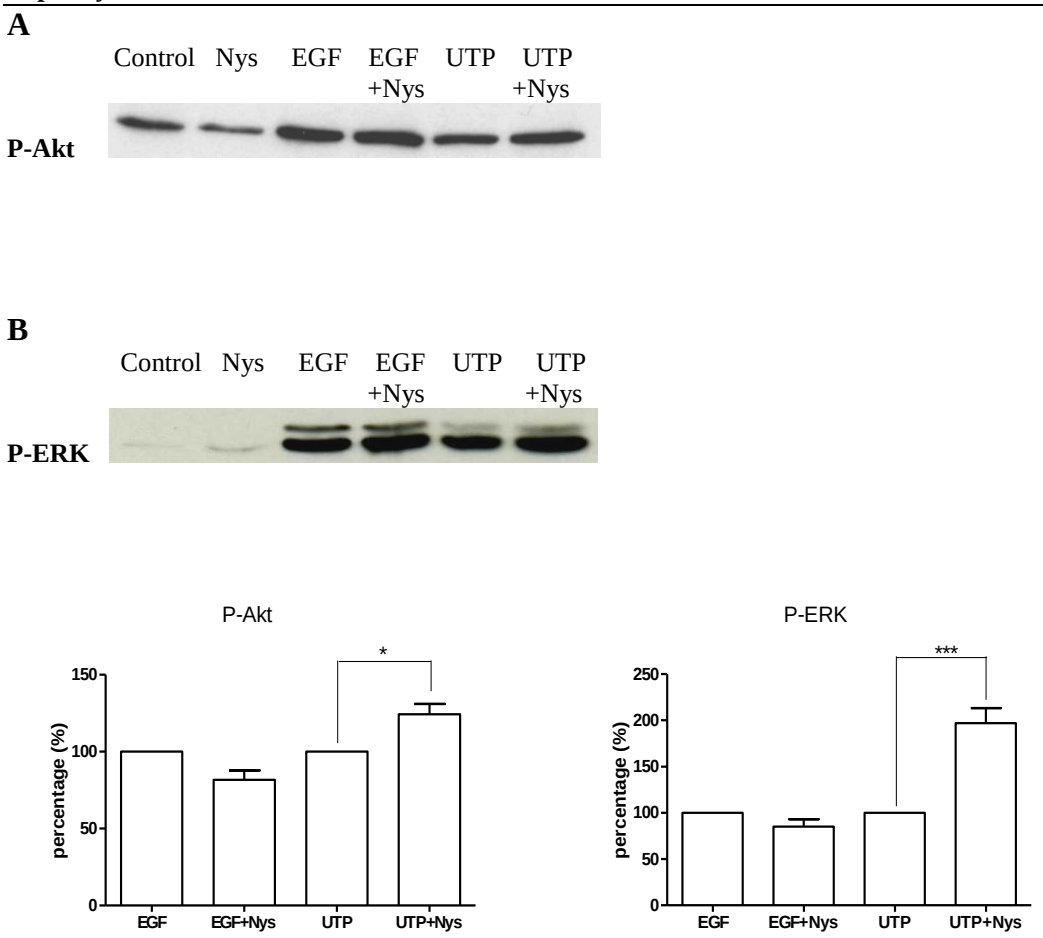


Figure 6.1 Effect of nystatin on EGF and UTP stimulation of A. Akt and B. ERK phosphorylation

Primary rat hepatocytes were cultured in WME supplemented with ITS and 10% FCS after plating for 4h, followed with 48h SF incubation. Cells were pre-incubated with 50mg/ml nystatin for 15min and then stimulated with 3nM EGF or 100μM UTP for 5min and 20min. Western blotting for phospho-ERK (20min) and phospho-Akt (5min) were measured. Data represented mean ± SEM (n=3) and were analysed by One-way ANOVA and post test was measured with Bonferroni's to compare all data sets using Graphpad Prism (*$p<0.05$, ***$p<0.001$). Western blots are representative of 3 experiments and statistic data are from 3 independent experiments.

6.2.1.2 Effect of the Clathrin-dependent internalisation on the EGF and UTP stimulation

The role of clathrin-coated vesicles in EGF/EGFR and UTP/$P2Y_2$ receptor internalisation was studied. In an attempt to investigate this, sucrose, an inhibitor of clathrin-coated vesicle-mediated endocytosis, was used. Since sucrose is a heavy compound and may deposit to form a high concentration solution at the bottom of the plate, in order to achieve the right concentration, two ways of adding sucrose were used. In one way, 0.9M sucrose was dissolved in WME and 1ml of resulting solution was added into cells which were in 1ml medium, to achieve a final 0.45M sucrose. The disadvantage of this method is that, without vigorous mixing (which itself stimulates the cell) the loading sucrose might results in cell being covered with 0.9M sucrose, twice the target concentration. Therefore, in the other way, 1ml of 0.45M sucrose was prepared and carefully layered into cells (in 1ml medium). Because the sucrose solution was heavier than medium (WME) it might form two levels of solution which at the bottom, near the cells, was 0.45M sucrose solution.

As shown in Figure 6.2, sucrose alone induced the phosphorylation of Akt and ERK and could not be used as an inhibitor. Moreover, either way of adding sucrose to hepatocytes provided very similar results.

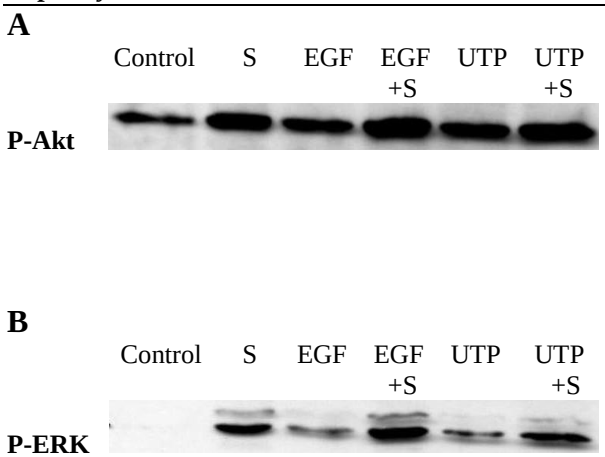


Figure 6.2 Effect of Sucrose on EGF and UTP stimulation of A. Akt and B. ERK phosphorylation

Primary rat hepatocytes were cultured in WME supplemented with ITS and 10% FCS after plating for 4h, followed with 48h SF incubation. Cells were pre-incubated with 0.45M sucrose for 30min and then stimulated with 3nM EGF or 100μM UTP for 5min and 20min. Western blotting for phospho-ERK (20min) and phospho-Akt (5min) were measured. Data are representative of 3 independent experiments.

Dynamin is essential for the formation and activation of clathrin-coated pits (Schmid, 1997; McNiven, 1998), therefore, clathrin-dependent internalisation was blocked by expressing Dn-dynamin. The Dn-dynamin encoded adenovirus was incubated with the cells for 16h, and the cells were further incubated for at least 8h without adenovirus to allow expression of Dn-dynamin before stimulation.

Figure 6.3 shows that the inhibition of the clathrin-dependent internalisation significantly reduced EGF stimulation of ERK phosphorylation. This was, however, only a partial effect. In the Dn-dynamin expressing hepatocytes, EGF stimulation of Akt phosphorylation was also reduced, although the response was not statistically significant. Furthermore, UTP stimulation of Akt phosphorylation was not affected by the Dn-dynamin treatment, while ERK response was reduced, although it was not significant.

The effect of inhibition of clathrin-dependent internalisation by Dn-dynamin on the EGF stimulation in hepatocytes was further studied. As shown in Figure 6.4A, EGF induced EGFR phosphorylation was significantly increased, when clathrin-dependent internalisation was inhibited. EGF stimulation of Akt phosphorylation was not affected by inhibition of clathrin-dependent internalisation after 5min, however, the response decreased to 60% ± 14% after 30min (Figure 6.4B). In Dn-dynamin expressing rat hepatocytes, EGF stimulation of ERK phosphorylation was decreased to 81% ± 6% and 77% ± 1% at 5min and 30min exposure of the cells to agonist respectively (Figure 6.4C).

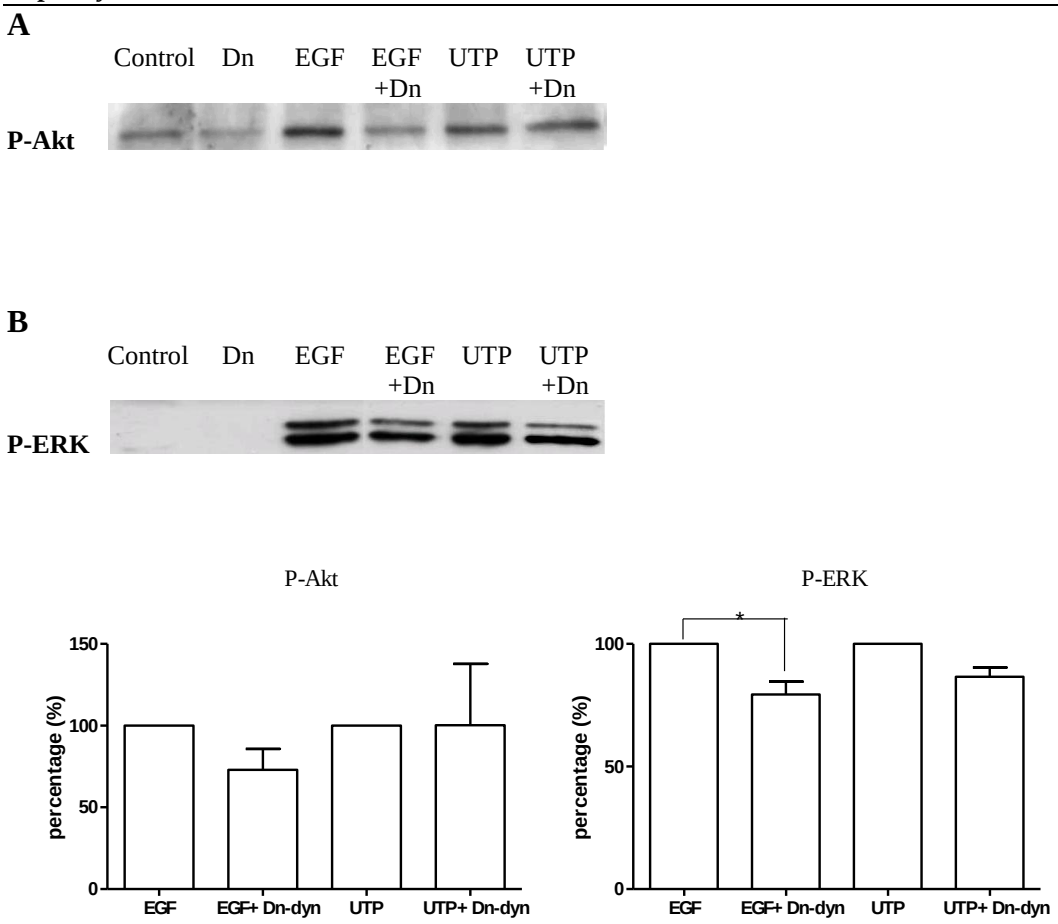
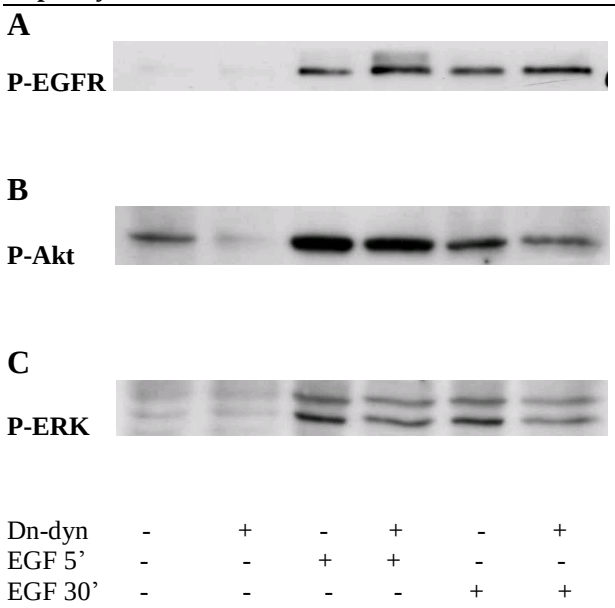


Figure 6.3 Effect of Dn-dynamin on the EGF and UTP stimulation of A. Akt and B. ERK phosphorylation

Hepatocytes were cultured for 4h in WME supplemented with ITS and 10% FCS, which were then replaced with SF WME containing encoding kinase-dead Dn-dynamin adenovirus for 16h and further incubated for 32h without adenovirus to allow the protein expression before stimulation with 3nM EGF or 100μM UTP for 5min or 20min. Western blotting of phospho-ERK (20min) and phospho-Akt (5min) were measured. Data represented mean ± SEM (n=3) and were analysed by One-way ANOVA and post test was measured with Bonferroni's to compare all data sets using Graphpad Prism (*$p<0.05$). Western blots are representative of 3 independent experiments and statistic data are from 3 independent experiments.



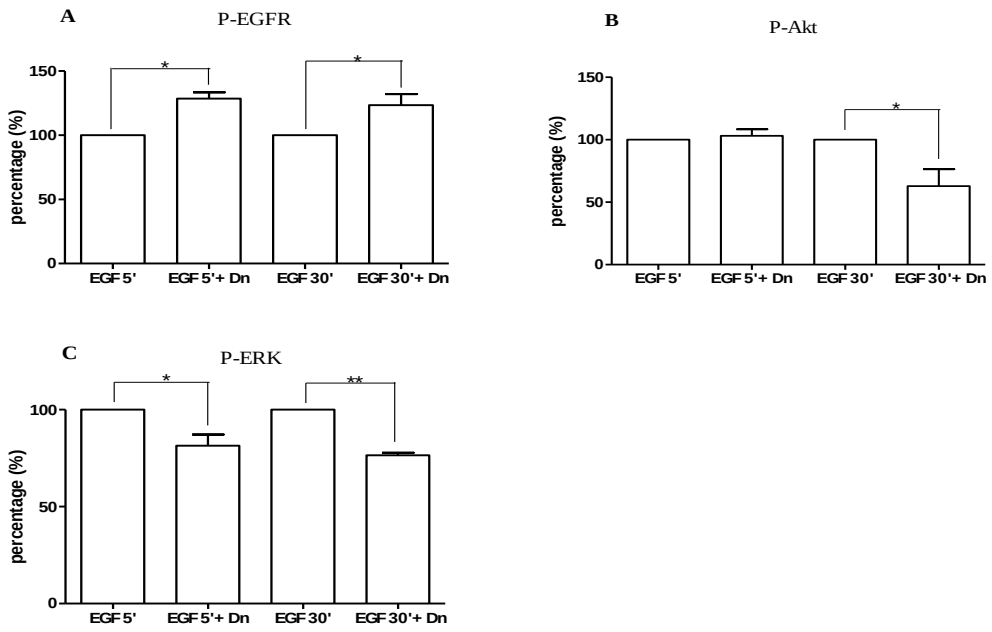


Figure 6.4 Effect of Dn-dynamin on the EGF stimulation of A. EGFR, B. Akt and C. ERK phosphorylation

Hepatocytes were cultured for 4h in WME supplemented with ITS and 10% FCS, which were then replaced with SF WME containing encoding kinase-dead Dn-dynamin adenovirus for 16h and further incubated for 32h without adenovirus to allow the protein expression before stimulation with 3nM EGF for 5min and 30min. Western blotting of phospho-EGFR, phospho-ERK and phospho-Akt were measured. Data represented mean ± SEM (n=3) and were analysed by One-way ANOVA and post test was measured with Bonferroni's to compare all data sets using Graphpad Prism (*$p<0.05$, **$p<0.01$). Western blots are representative of 3 independent experiments and statistic data are from 3 independent experiments.

Since EGF stimulation is at least partially clathrin-dependent, as suggested in Figure 6.3 and Figure 6.4, the location of EGF and EGFR after ligand stimulation were further studied. Cells were incubated with 488-EGF and Alexa-546 fluorescence conjugated transferrin (transferrin-546), a marker for clathrin-dependent internalisation, for 30min to visualise whether the internalisation of EGF is clathrin-dependent in hepatocytes. Furthermore, Dn-dynamin expressing cells were incubated with 488-EGF and transferrin-546 to examine the effect of Dn-dynamin on the EGF and transferrin internalisation. The green fluorescence was from 488-EGF, red signal was from transferrin-546 and the yellow signal was the combined green and red fluorescence.

Figure 6.5A shows that both EGF (green) and transferrin (red) internalised after 30min stimulation. The co-location (yellow) of EGF and transferrin suggested that EGF internalisation is clathrin-dependent in hepatocytes. It shows that the inhibition of the formation of clathrin-coated vesicles by expression of Dn-dynamin in hepatocytes effectively blocked the EGF and transferrin internalisation (Figure 6.5B). In order to ask whether the internalisation of the receptor (rather than the ligand) was blocked, the EGFR was visualised by confocal immunohistochemistry using an anti-EGFR antibody. As shown in Figure 6.6, treatment of cells expressing Dn-dynamin blocked the EGF-mediated EGFR internalisation.

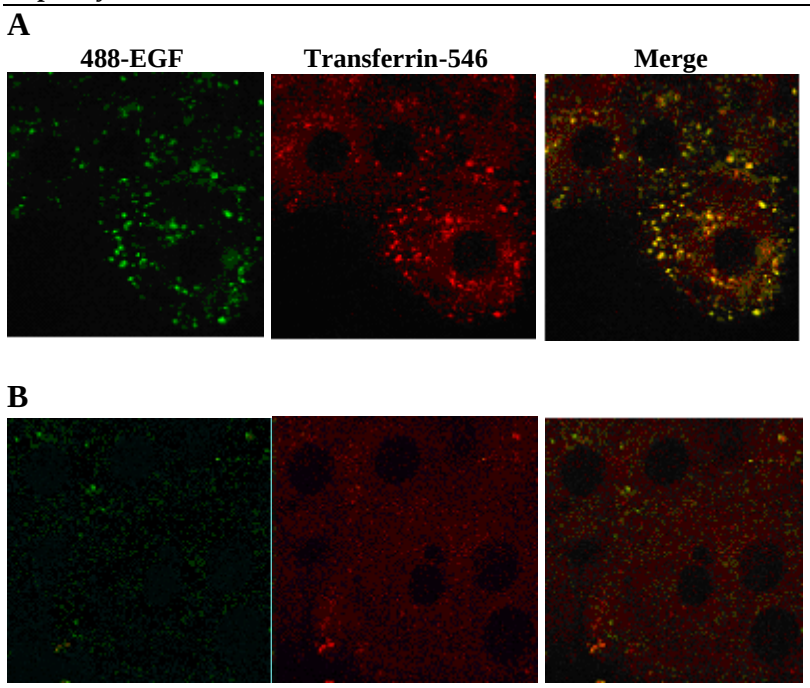


Figure 6.5 Location of EGF and transferrin after 30min stimulation in A. normal primary rat hepatocytes and B. Dn-dynamin expressed hepatocytes

Hepatocytes were cultured on a plastic coverslip for 4h in WME supplemented with ITS and 10% FCS, which were then replaced with SF WME for 24h. In Panel B, the replaced WME containing encoding kinase-dead Dn-dynamin adenovirus for 16h and further incubated for 8h without adenovirus to allow the protein expression. Then cells were stimulated with 60nM 488-EGF and 20μg/ml transferrin-546 for 30min. The reaction was stopped by fixing in 4% paraformadehyde at 4℃ for 15min. The coverslip was mounted onto a slide and images collected using a confocal microscope. Images are representative of 2 experiments.

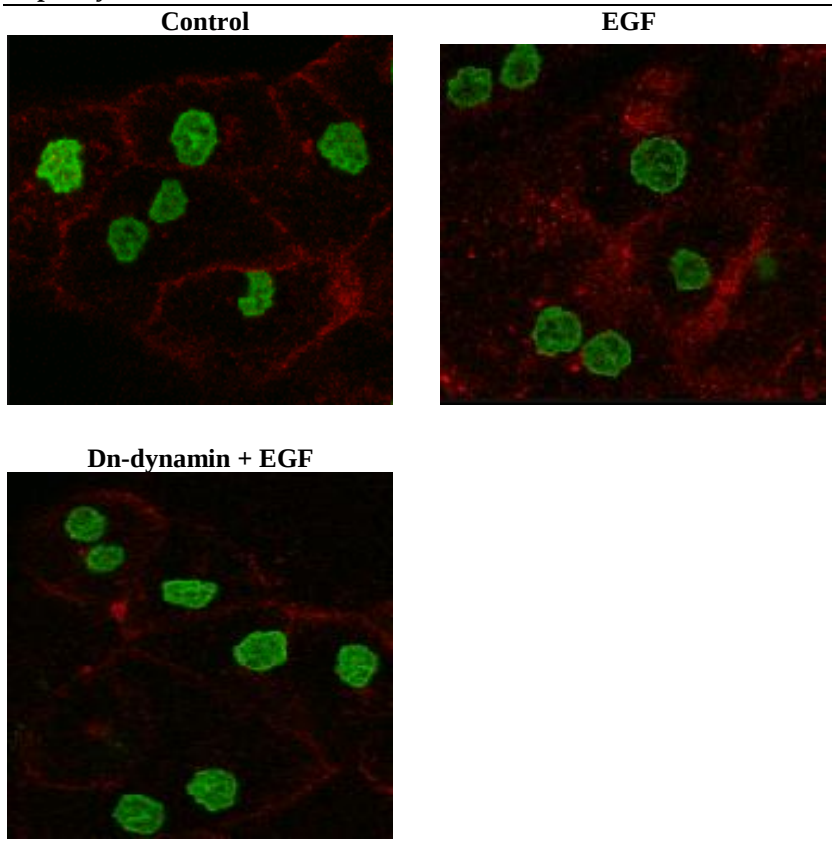


Figure 6.6 Effect of the Dn-dynamin on the ligand-mediated EGFR internalisation

Cells were cultured on a plastic coverslip in WME supplemented with ITS and 10% FCS for 4h after plating, followed with changed medium to SF and further incubated for 24h. The cells were stimulated with 60nM EGF for 30min. Then reaction was stopped by fixing the cells in 4% paraformadehyde at 4℃ for 15min, subsequently permeabilized in 0.1% Triton (in 1% BSA/PBS) for 2min on ice. After that cells were incubated in 10% donkey serum (in 2% BSA/PBS) at room temperature for 1h and then labelled by 1:50 anti-EGFR at 4℃ overnight. The next day, cells were applied with 1:200 donkey anti-rabbit cy5 at room temperature for 1h in the dark and the nucleus was labelled by incubation with 5mM SYTOX for 30s at room temperature. Finally the coverslip was mounted on a slide and images were collected using a confocal microscope. Images are representative of 3 experiments.

6.2.2 The study of the $P2Y_2$ receptor and EGFR location in primary rat hepatocytes

6.2.2.1 The location of the $P2Y_2$ receptors

The previous results (Figure 5.8 and Figure 5.9) have indicated that activated-$P2Y_2$ stimulated down-stream proteins on the cell surface and inhibition of receptor internalisation may increase the extent of stimulation. Lipofectamine-2000 or jetPEI^{TM}-Hepatocyte DNA Transfection Reagent (Polyplus Transfection) was used to transiently transfect cells to express green fluorescence protein (GFP) coupled $P2Y_2$ receptor. This enabled the location of the receptor to be visualised.

Figure 6.7A&C show the fluorescence control levels of primary rat hepatocytes. Figure 6.7B shows that incubation of the cells with lipofectamine-2000 and GFP-$P2Y_2$ plasmid for 48h resulted in the cells being transfected with GFP-$P2Y_2$ receptor. However, the GFP signal was detected over the entire cell and was not restricted to the cell surface. Transfection mediated by Reagent kit (Polypus Transfection) gave a similar result, as shown in Figure 6.7D. Therefore, P2Y2 receptor internalisation is not able to directly visualise and new method needs to be developed.

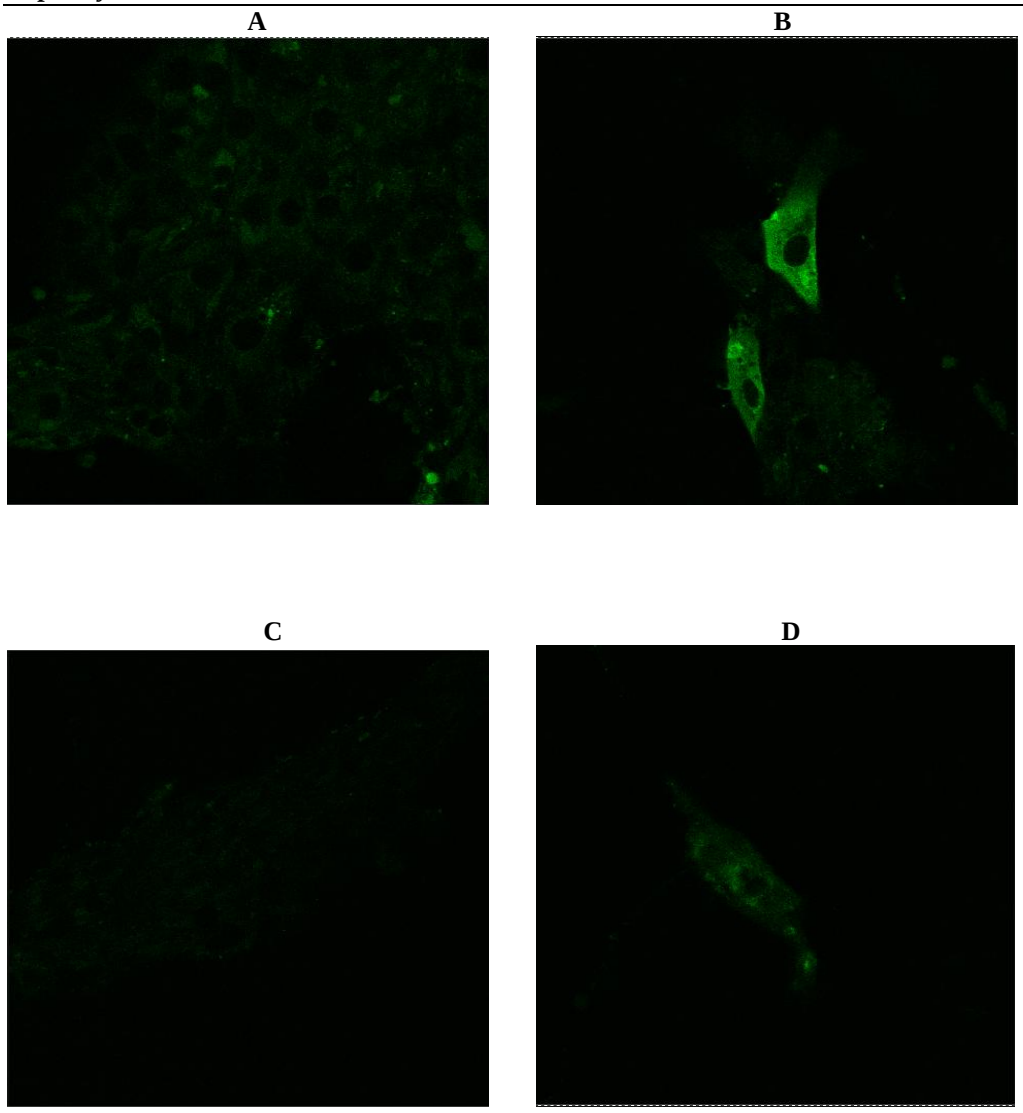


Figure 6.7 Confocal images of A. non-transfected cells, B. lipofectemin-2000 transfection of GFP-P2Y$_2$ receptor cells, C. non-transfection cells and D. jetPEI^{TM}-Hepatocyte DNA Transfection Reagent transfection of GFP-P2Y$_2$ receptor cells

Cells were cultured on a plastic coverslip in WME supplemented with ITS and 10% FCS for 4h after plating, followed with transfected of cells with GFP-P2Y$_2$ receptor plasmid by lipofectamine-2000 or jetPEI^{TM}-Hepatocyte DNA Transfection Reagent for 48h. The cells were then fixed in 4% paraformadehyde at 4℃ for 15min and the coverslip was then mounted on a slide. Images are representative of 2 independent experiments.

6.2.2.2 The internalisation of EGF and EGFR

6.2.2.2.1 The location of EGF after stimulation

The previous results (Figure 5.8) suggested the possibility that internalisation was necessary for EGF stimulation of ERK and Akt phosphorylation. In order to further study EGF internalisation, 488-EGF was used in place of EGF to stimulate the cells and visualise the location of EGF. The nucleus was labelled with PI.

Figure 6.8 presents a series of images of EGF location, captured with the confocal microscope, in hepatocytes with different treatments. Panel A shows the fluorescence in the absence of 488-EGF; hepatocyte auto-fluorescence signal was removed with appropriate setting of the confocal microscope. The other images were measured with the same setting to eliminate the effect of hepatocyte auto-fluorescence. In the experiments, the position of the optical slices (Z-dimension) was set to maximise the signal of the nucleus, which was counterstained with PI (Red) to ensure that the images collected were within the body of the cell. Panel B shows the location of EGF in the cells, which were exposed to 488-EGF for 30min. It presents a typical example of the punctuate appearance of Alexa-488 fluorescence in the interior of the cell, with no labelling of the cell surface. The fluorescence was completely lost when cells were first incubated with excess unlabelled EGF for 5min before exposure to 488-EGF (Panel C). There was also no Alexa-488 fluorescence detected in the cells which were pre-incubated with con A before stimulation (Panel D).

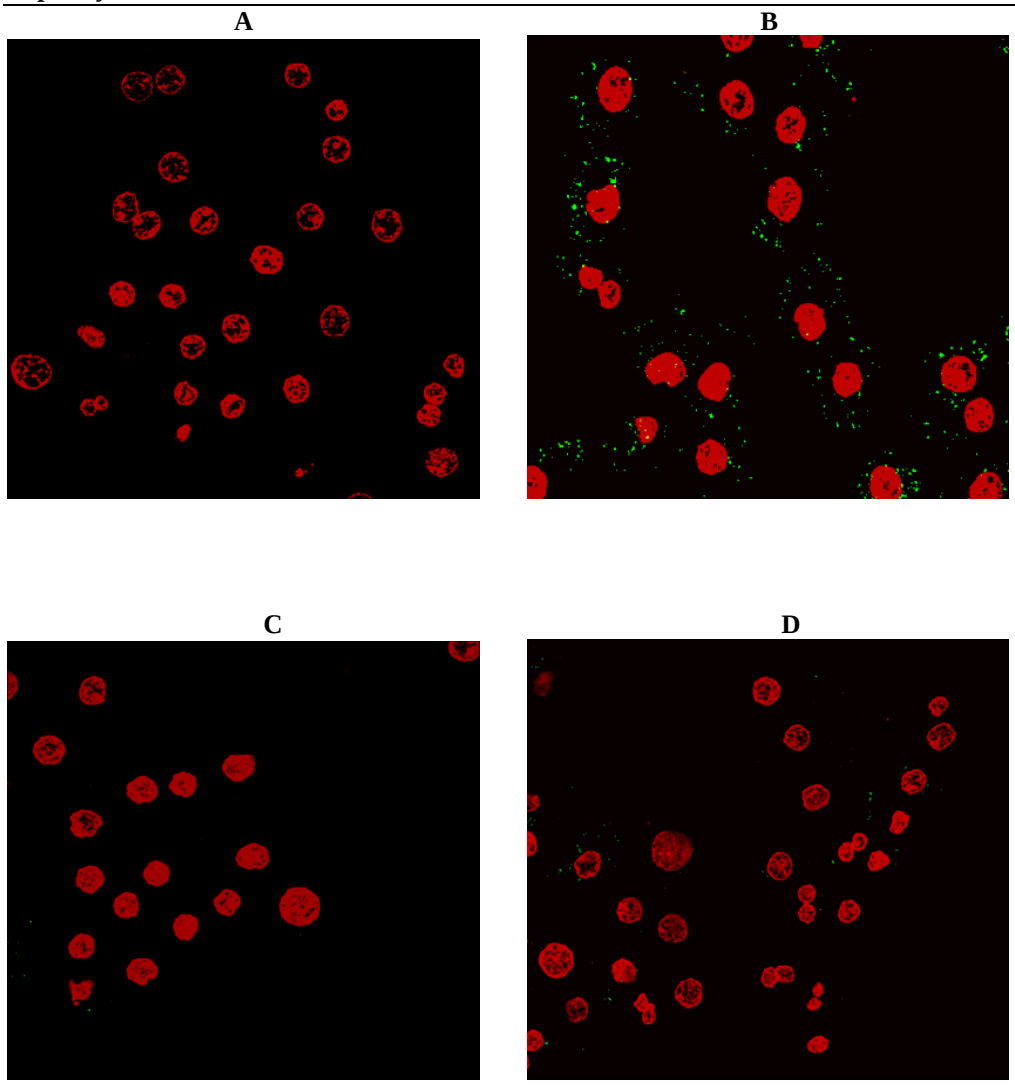


Figure 6.8 Confocal images of 488-EGF location in hepatocytes in different conditions: A. without 488-EGF stimulation; B.488-EGF stimulation for 30min; C. pre-incubation with cold-EGF for 5min and then stimulation with 488-EGF for 30min; D. pre-incubation with con A for 15min and then stimulation with 488-EGF for 30min

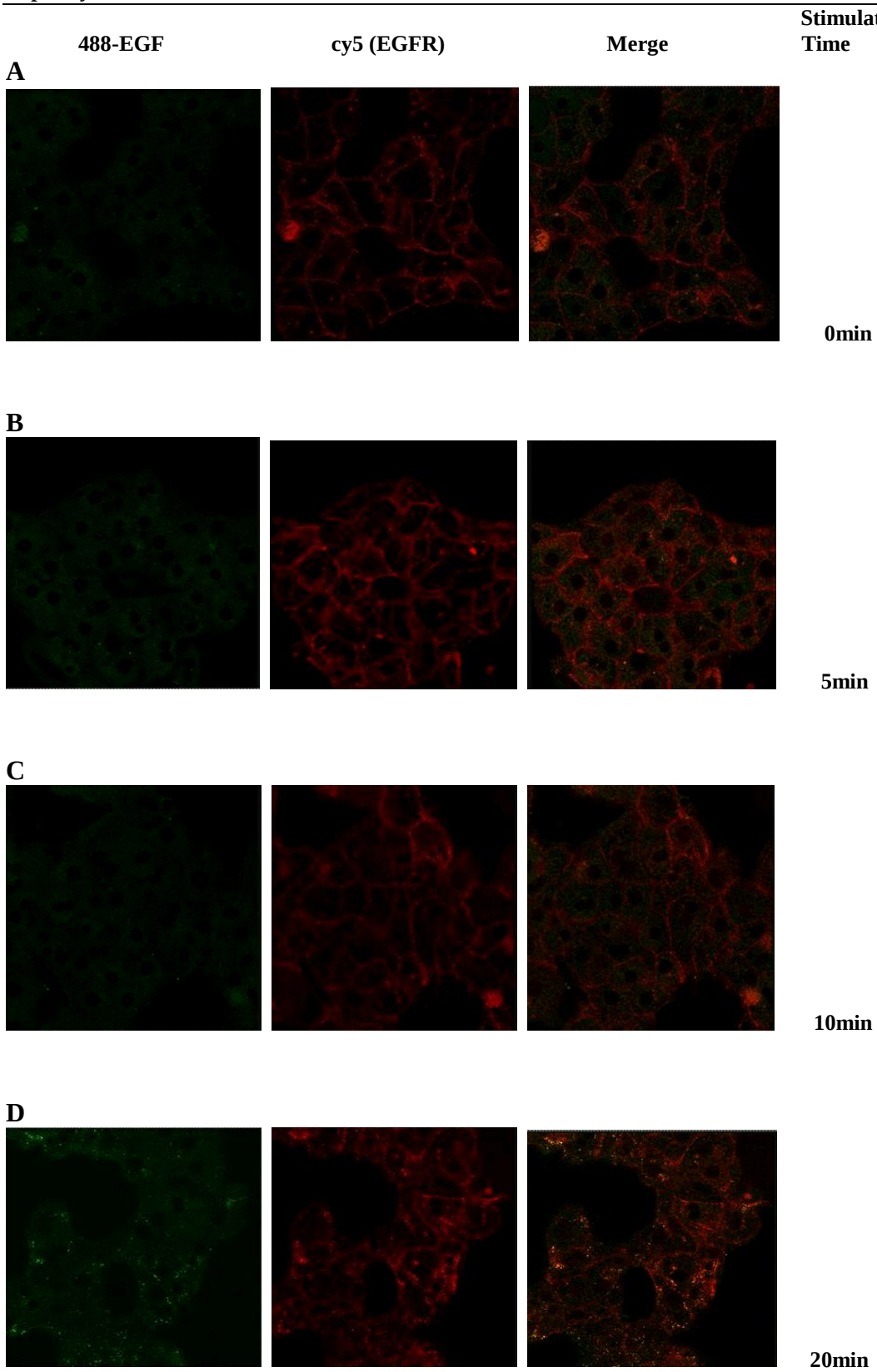
Cells were cultured on a plastic coverslip in WME supplemented with ITS and 10% FCS for 4h after plating, followed with changed medium to SF and further incubated for 24h. Cold-EGF (60nM) was added 5min before while 250μM con A were added 15min before exposure of cells to 60nM 488-EGF for 30min. The reaction was stopped by fixing cells in 4% paraformadehyde at 4℃ for 15min; subsequently cells were incubated with 50μg/ml PI with 10μg/ml RNAse for 30min at 37℃ to label the nucleus. After that washed extracellular PI off with PBS and the coverslip was mounted on a slide. Images are representative of 3 independent experiments.

6.2.2.2.2 The location of the EGF and EGFR at different time points after stimulation

Figure 6.8 shows that exposure of cells to EGF resulted in EGF internalisation after 30min. Experiments were then designed to investigate when the internalisation starts and duration of the process. Cells were stimulated with 488-EGF for different times (up to 1h) and the location of the EGFR after ligand stimulation was visualised by immunocytochemistry, as described in Chapter 2, Section 2.2.6. The green fluorescence was from 488-conjugated EGF, the red signal was from cy5-binding to the EGFR and the orange image was the combined green and red fluorescence.

The Figure 6.9A shows the image of the control cells (un-stimulated), which was used to set the sensitivity of the confocal microscope. Figure 6.9B-F show the location of EGF and EGFR in the cells that were exposed to ligand for 5min, 10min, 20min, 30min and 1h respectively. Internalisation of EGF and EGFR was not clearly evident during the first 10min. However, a comparison images at 0min, 5min and 10min shows a pro-increase thickening of the EGFR labelled periphery of the cells. In Panel A, the periphery was sharp and thin, while, in Panel B and C, this became less sharp and thicker, consistent with movement of the EGFR into the cells. EGF and EGFR internalisation were clearly visualised as bright punctuate green (EGF) or red (EGFR) spots after 20min, as shown in Panel D. By 20min, most of the green and red fluorescence signals overlapped inside the cells, which indicated the association of the EGF and EGFR. When the cells were stimulated by EGF for 30min (Panel E), a large number of co-located green and red signals were again detected inside of the cells. Interestingly, Panel F shows that after 1h exposure, there were relatively few co-located EGF and EGFR signals detected inside of the cells. Most green fluorescence signals

were individually located inside of the cell and remained green, when the images (488-EGF and cy5-receptor) were merged. There only a few red fluorescence signals and formed as cluster were detected, most of which were captured on or near the cell surface.


488-EGF
cy5 (EGFR)
Merge
Stimulation Time
A
0min
B
5min
C
10min
D
20min

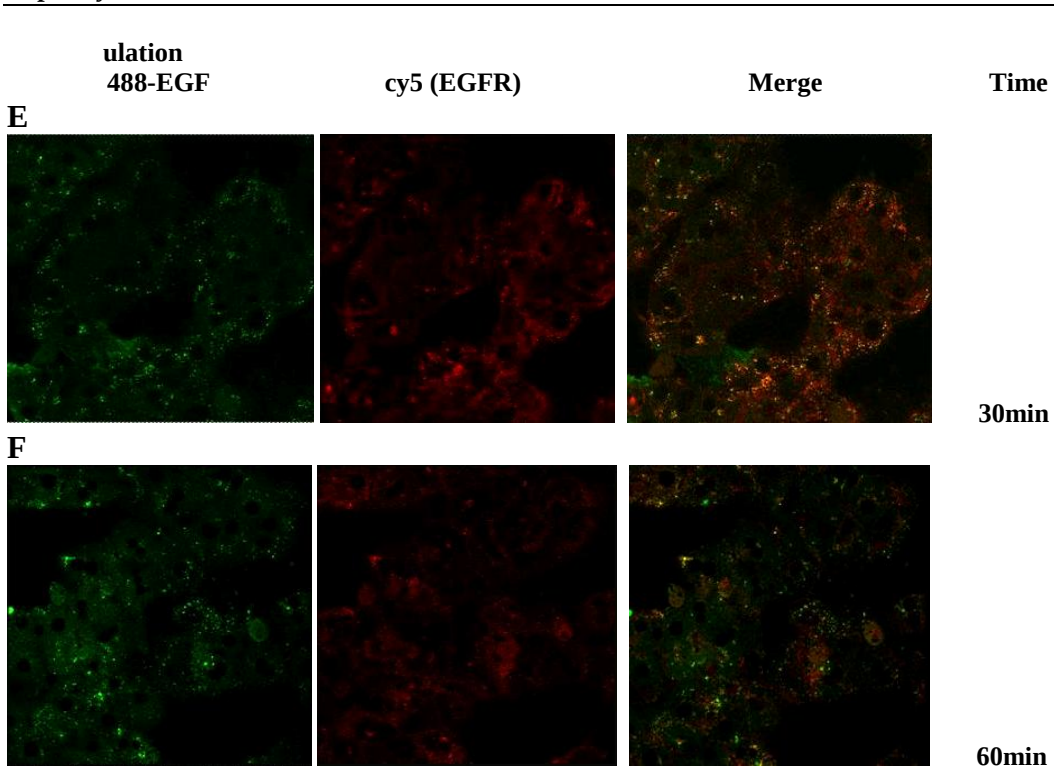


Figure 6.9 Time course of the EGF and EGFR location after ligand stimulation for A. 0min (un-stimulated), B. 5min, C. 10min, D. 20min, E. 30min and F. 60min

Cells were cultured in WME supplemented with ITS and 10% FCS on a plastic converslip for 4h after plating, followed with changed medium to SF and further incubated for 24h. The cells were stimulated with 60nM 488-EGF for different times, as indicated. Then reaction was stopped by fixing the cells in 4% paraformadehyde at 4℃ for 15min, subsequently permeabilised in 0.1% Triton (in 2% BSA/PBS) for 2min on ice. After that cells were incubated in 10% donkey serum (in 2% BSA/PBS) at room temperature for 1h and then labelled by 1:50 anti-EGFR at 4℃ overnight. The next day, cells were labelled by 1:200 donkey anti-rabbit cy5 at room temperature for 1h in the dark. Finally the coverslip was mounted on a slide and images were captured by confocal microscope. Images are representative of 3 independent experiments.

6.2.2.2.3 The location of the phospho-EGFR (Tyr1173) at different time after stimulation

The previous results (Figure 6.9) have shown that the EGFR was translocated into the cell after ligand stimulation. In order to study whether the internalised EGFR is activated, the time course of phospho-EGFR location was also investigated. Cells were stimulated with EGF for different times (up to 1h) and the phospho-EGFR was visualised using immunocytochemistry. The nucleus was labelled with SYTOX ® Green nucleic acid stain (SYTOX). The green fluorescence was from SYTOX labelled nucleus and the red signal was from cy5-binding phospho-EGFR.

Figure 6.10A shows the background image as before. Panel B-E show a series of images of the location of phospho-EGFR at different times after stimulation. Phospho-EGFR was detected after 5min (the first time point of the experiment) and was located within the nucleus as shown in Panel B. The level of the phosphorylated EGFR increased and reached a maximum in this experiment after 30min. Panel D shows that after 30min stimulation, the signal of phospho-EGFR was seen in most cells. The accumulation of signal then decreased, but could still be detected in the nucleus after 60min, as shown in Panel E.

Further evidence of EGF stimulation of the translocation of phospho-EGFR into the nucleus was provided by preparing cell fractionation and western blotting for either cytoplasmic or nuclear component. Cells were exposed to EGF for 30min and cytoplasmic and nuclear solutions were separated by using NE-PER Nuclear and

Cytoplasmic Extraction Reagents after lysis. EGFR and phospho-EGFR was measured by western blotting.

As shown in Figure 6.11A, EGF stimulated phospho-EGFR was located in both cytoplasm and nucleus in primary rat hepatocytes. Furthermore, exposure of the hepatocytes to EGF resulted in a clear increase of EGFR in nucleus compared to control (un-stimulated) cells (Figure 6.11B). This indicates EGF induced translocation of EGFR from cytoplasm into the nucleus. Both the phospho-EGFR and the pan-EGFR blots in Figure 6.11 show that EGF stimulation resulted in the enhanced appearance of double bands, with the upper band appearing more strongly on stimulation. This is consisted with EGF-stimulated hyperphosphorylation of the EGFR.

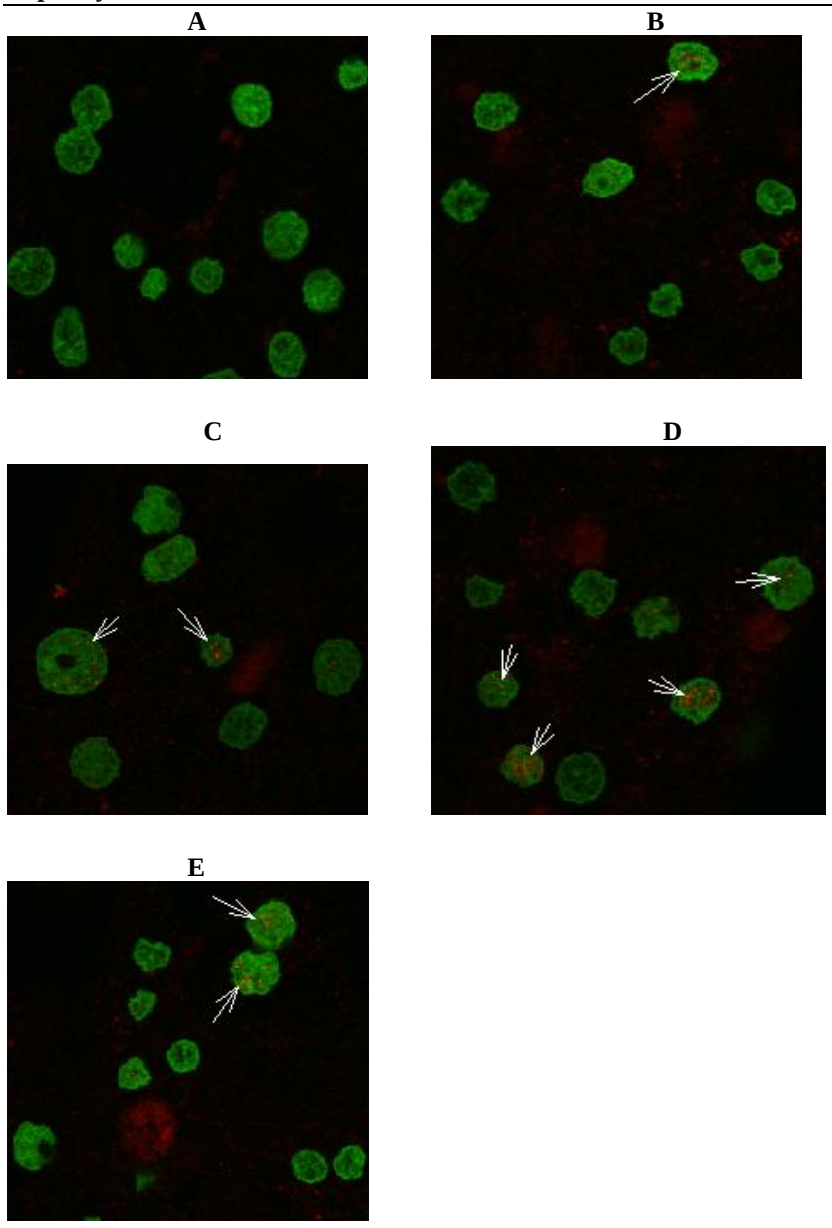


Figure 6.10 Time course of the phospho-EGFR (Tyr1173) location after ligand stimulation for A. 0min (un-stimulated), B. 5min, C. 15min, D. 30min, E. 60min

Cells were cultured in WME supplemented with ITS and 10% FCS on a plastic coverslip for 4h after plating, followed with changed medium to SF and further incubated for 24h. The cells were stimulated with 60nM EGF for different times, as indicated. Then reaction was stopped by fixing the cells in 4% paraformadehyde at 4℃ for 15min, subsequently permeabilised in 0.1% Triton (in 2% BSA/PBS) for 2min on ice. After that cells were incubated in 10% donkey serum (in 2% BSA/PBS) at room temperature for 1h and then labelled by 1:50 anti-P-EGFR (tyr1173) at 4℃ overnight. The next day, cells were labelled by 1:200 donkey anti-rabbit cy5 at room temperature for 1h in the dark and the nucleus was labelled by incubation with 5mM SYTOX for 30s at room temperature. Finally the coverslip was mounted on a slide and images were captured by confocal microscope. Images are representative of 3 independent experiments.

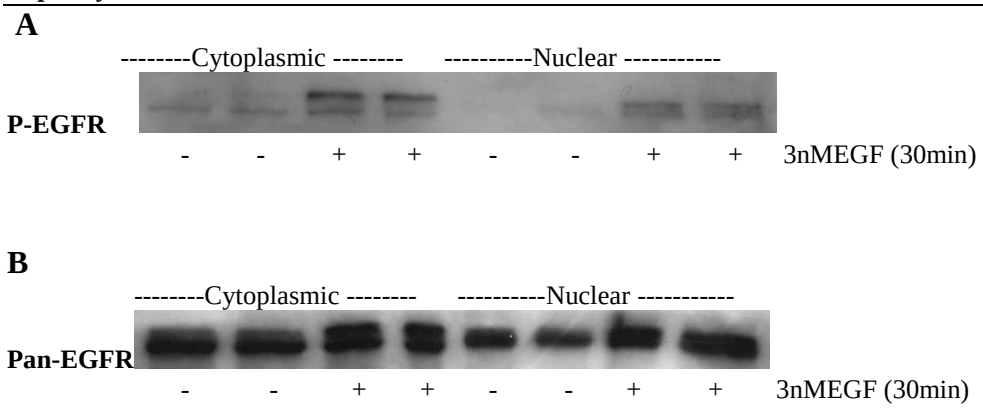


Figure 6.11 Effect of EGF on cytoplasmic and nuclear A. phospho-EGFR and B. EGFR in primary rat hepatocytes

Primary rat hepatocytes were cultured in WME supplemented with ITS and 10% FCS after plating for 4h, followed with 48h SF incubation before being exposed to 3nM EGF for 30min as labelled. Cytoplasmic and nuclear fractions were separated by using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific Pierce Protein Research Products) after lysis, and western blotting of phospho-EGFR and pan-EGFR were measured. Western blots are representative of 2 independent experiments.

6.2.3 Effect of internalised EGF/EGFR on the phosphorylation of Akt, ERK and cell cycle progression

6.2.3.1 The accumulation of internalised EGF/EGFR by blocking the activation of EGFR

The previous results have shown that receptor internalisation is important for EGF stimulation of primary rat hepatocytes. AG1478 abolished EGF stimulation of the phosphorylation of the EGFR and cell cycle progression in hepatocytes (Chapter 3). In order to investigate whether the internalisation of the EGFR was dependent on the phosphorylation of the receptor, cells were incubated with AG1478 for 15min before stimulation with 488-EGF and the location of EGF, EGFR and phospho-EGFR were visualised.

In Figure 6.12, the green fluorescence signal was 488-EGF, while the red signal presented PI-labelled nucleus. Panel A shows that the incubation of the cells with AG1478 did not affect the fluorescence background of the hepatocytes. In Panel B EGF was internalised after 30min stimulation. When the cells were pre-incubated with AG1478 to inhibit the phosphorylation of EGFR, the EGF was also detected inside of the cells, as shown in Panel C. Furthermore, the inhibition of EGFR phosphorylation appeared to cause the accumulation of EGF in hepatocytes. There was more punctuate fluorescence within the cells, which were treated with AG1478 followed by stimulation (Panel C) than cells incubated with EGF but no inhibitor (Panel B).

In Figure 6.13 the green fluorescence signal was 488-EGF and the red fluorescence signal came from cy5-binding EGFR antibody and the yellow signal presented the co-

located EGF (green) and EGFR (red). Panel A shows that treatment with AG1478 alone did not affect the EGFR location and also did not affect the fluorescence background. Exposure of cells to EGF for 30min resulted in the internalisation of both the EGF and EGFR. Furthermore, most ligand was associated with its receptor (yellow signal) at this time, as presented in Panel B, suggested the EGF and EGFR forming a complex. Moreover, cy5 red signals were detected at cell surface which suggested that some receptor were recycled to the membrane after internalisation or did not move from the surface while stimulation. However, when the cells were pre-treated with AG1478 before EGF stimulation there was no inhibition on EGF and EGFR complex internalisation, as shown in Panel C. Moreover, no red signal detected on the cell surface suggested that AG1478 inhibited the recycling of the receptor or caused the accumulation of the receptor inside cells.

Figure 6.14 shows the effect of AG1478 on the EGF induced phospho-EGFR translocation into the nucleus in hepatocytes. The images show that EGF-induced nuclear EGFR phosphorylation and/or phospho-EGFR translocation in the nucleus was inhibited by AG1478.

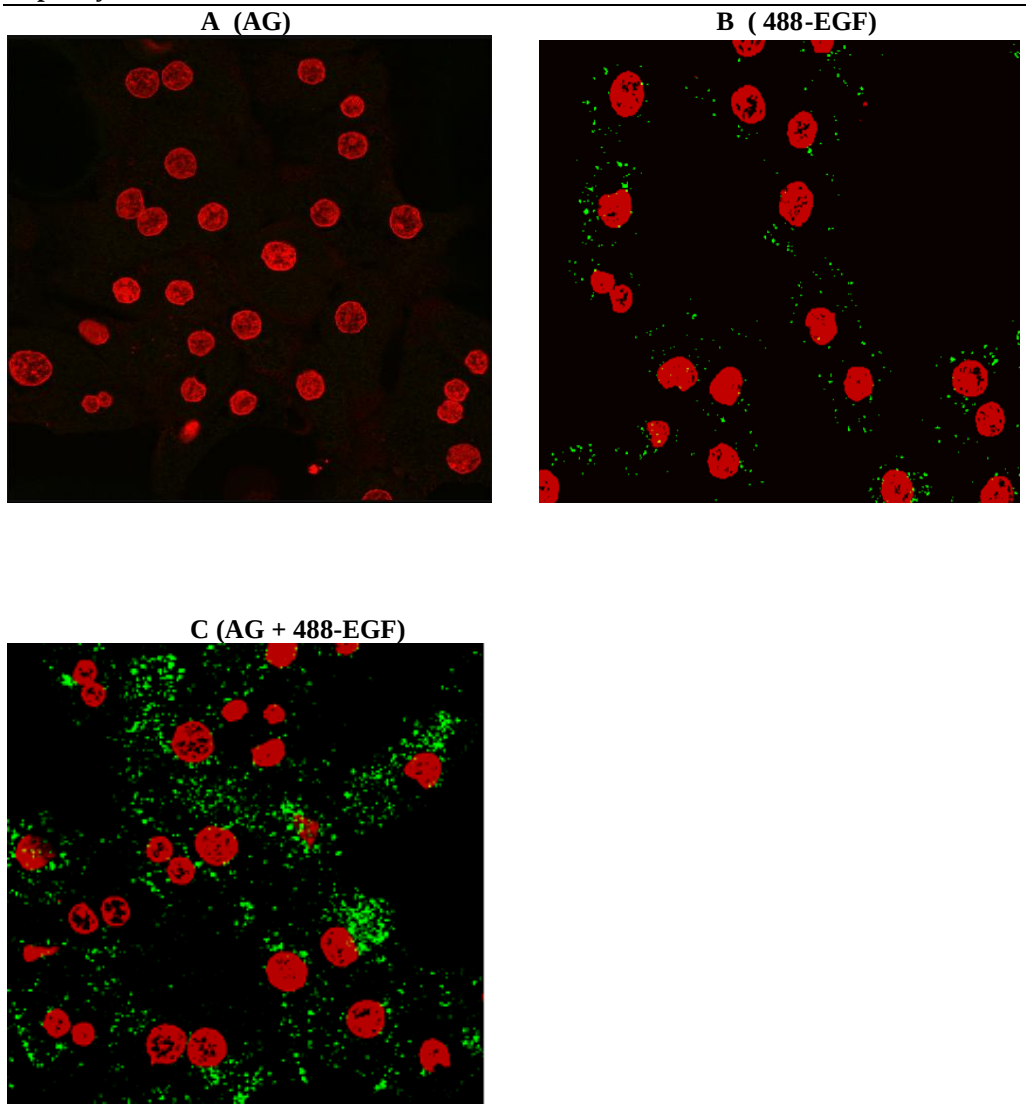


Figure 6.12 Effect of AG1478 on the EGF internalisation in hepatocytes

Cells were cultured in WME supplemented with ITS and 10% FCS on a coverslip for 4h after plating, followed with changed medium to SF and further incubated for 24h. 300nM AG1478 was added 15min before exposure of the cells to 60nM 488-EGF for 30min. Then cells were fixed in 4% paraformadehyde at 4℃ for 15min, subsequently incubated with 50μg/ml PI and 10μg/ml RNAse for 30min at 37℃ to label the nucleus. Cells were washed with PBS to remove extra PI and the coverslip was mounted on a slide. Images are representative of 3 independent experiments.

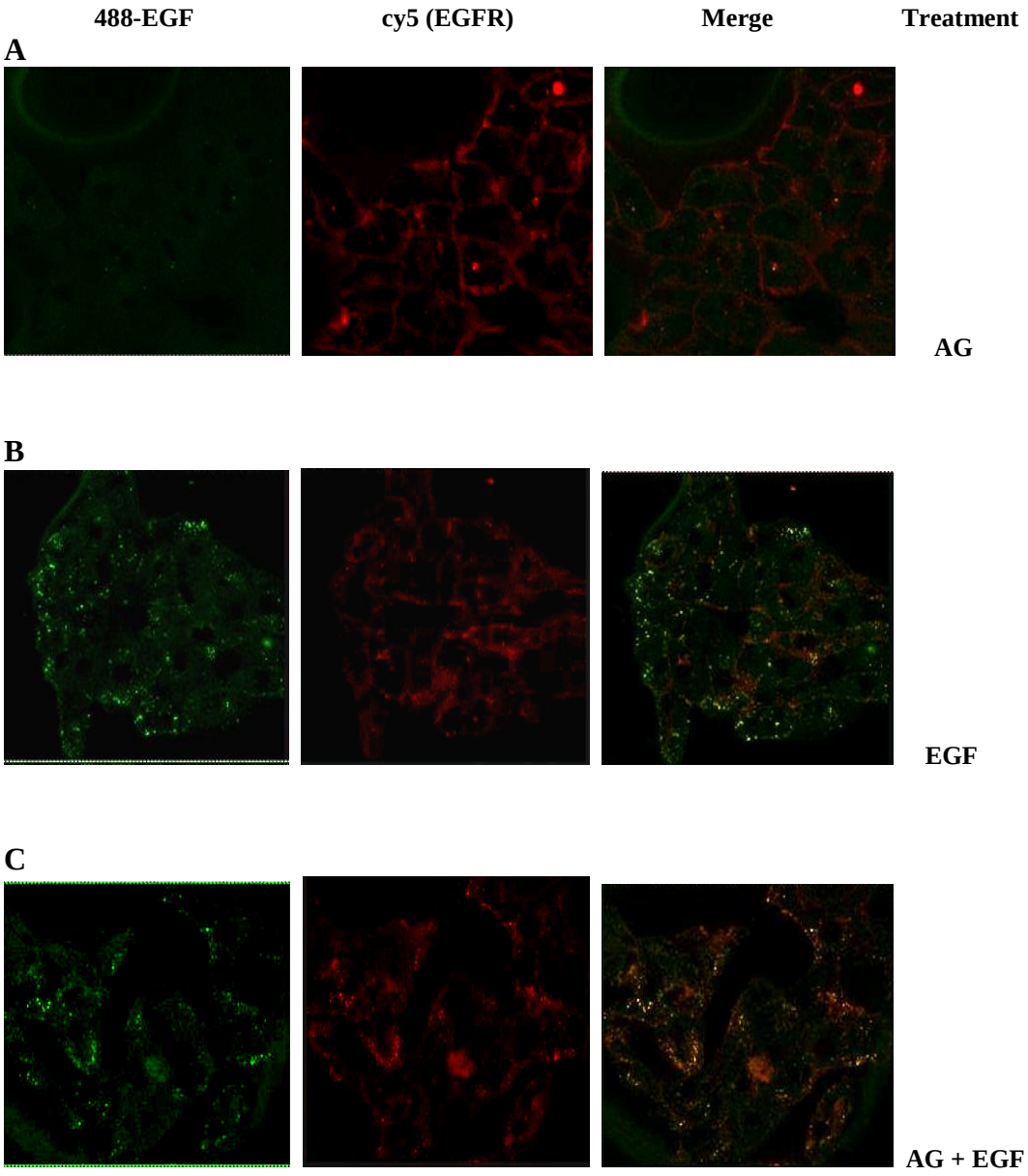


Figure 6.13 Effect of AG1478 on EGF and EGF-induced EGFR internalisation

Cells were cultured in WME supplemented with ITS and 10% FCS on a coverslip for 4h after plating, followed with changed medium to SF and further incubated for 24h. 300nM AG1478 was added 15min before exposure of the cells to 60nM 488-EGF for 30min. Then reaction was stopped by fixing the cells in 4% paraformadehyde at 4℃ for 15min, subsequently permeabilized in 0.1% Triton (in 2% BSA/PBS) for 2min on ice. After that cells were incubated in 10% donkey serum (in 2% BSA/PBS) at room temperature for 1h and then labelled by 1:50 anti-EGFR at 4℃ overnight. The next day, 1:200 donkey anti-rabbit cy5 was applied at room temperature for 1h in the dark. Finally the coverslip was mounted on a slide and images were captured by confocal microscope. Images are representative of 3 independent experiments.

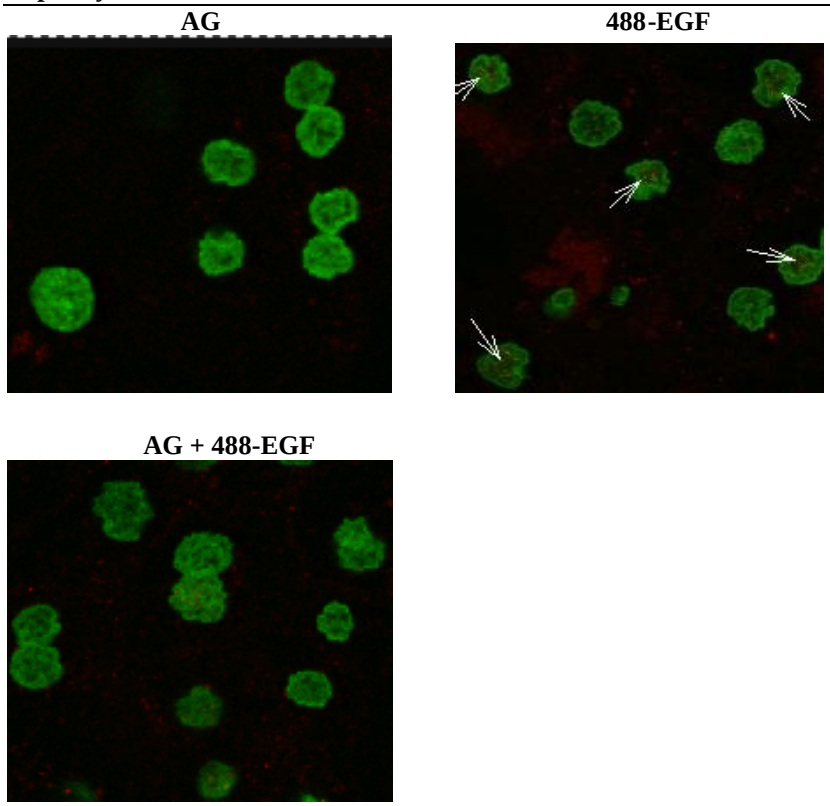


Figure 6.14 Effect of AG1478 on EGF-induced phospho-EGFR internalisation

Cells were cultured in WME supplemented with ITS and 10% FCS on a coverslip for 4h after plating, followed with changed medium to SFand further incubated for 24h. 300nM AG1478 was added 15min before exposure of the cells to 60nM 488-EGF for 30min. Then reaction was stopped by fixing the cells in 4% paraformadehyde at 4℃ for 15min, subsequently permeabilized in 0.1% Triton (in 2% BSA/PBS) for 2min on ice. After that cells were incubated in 10% donkey serum (in 2% BSA/PBS) at room temperature for 1h and then labelled by 1:50 anti-P-EGFR (tyr1173) at 4℃ overnight. The next day, 1:200 donkey anti-rabbit cy5 was applied at room temperature for 1h in the dark and the nucleus was labelled by incubation of the cells with 5mM SYTOX for 30s at room temperature. Finally the coverslip was mounted on a slide and images were captured by confocal microscope. Images are representative of 3 independent experiments.

6.2.3.2 The effect of EGFR activation on ligand-induced receptor internalisation

In order to visualise the location of EGF, 60nM 488-conjugated EGF was used to stimulate the cells in previous experiments as fluorescence signal could not be detected at lower concentration. This concentration was 20 times higher than the concentration used in other experiments (western blotting, [^{3}H]-thymidine and MTT assay) to stimulate hepatocytes. Therefore, it was necessary to study whether the internalisation of EGF, EGFR and phospho-EGFR, also happened on exposure of cells to low concentrations (3nM) of EGF as used in other experiments. Furthermore, the effect of the inhibition of the EGFR activation on the EGF/EGFR internalisation was also investigated. Therefore, cells were exposed to 3nM EGF with/without AG1478, and the location of EGFR was investigated.

Figure 6.15 shows that low concentrations of EGF also induced EGFR internalisation after exposure of the cells to EGF for 30min. Furthermore, AG1478 again did not inhibit the EGF-mediated the EGFR internalisation in hepatocytes. This suggested that the receptor internalisation was ligand dependent and not requiring receptor activation in primary rat hepatocytes.

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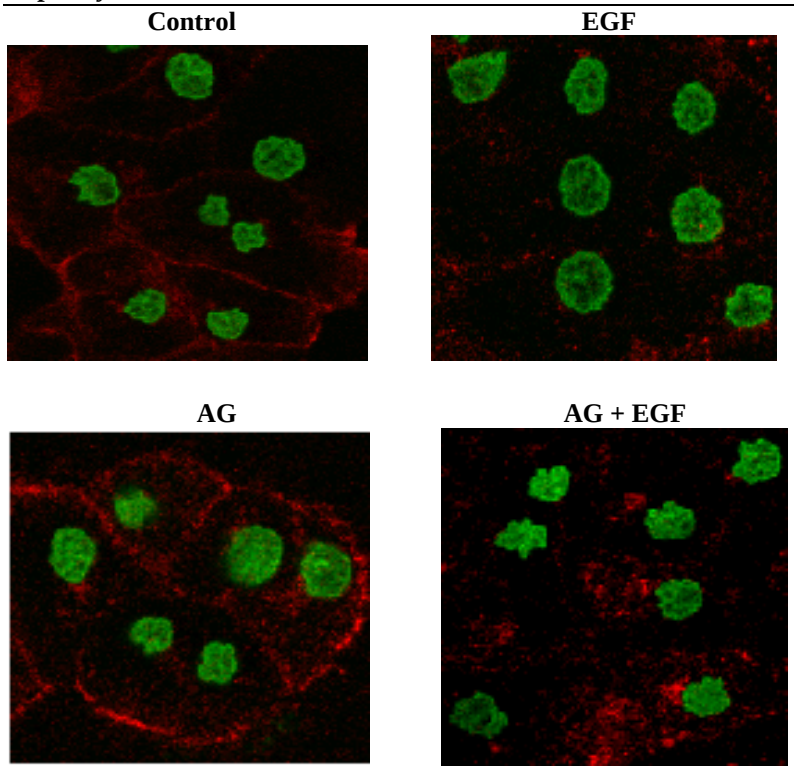


Figure 6.15 The location of the EGFR in the low concentration (3nM) of EGF-stimulated cells and effect of AG1478 on this concentration of EGF-mediated EGFR internalisation

Cells were cultured in WME supplemented with ITS and 10% FCS for 4h after plating, followed with changed medium to SF and further incubated for 24h. 300nM AG1478 was added 15min before stimulation of the cells with 3nM EGF for 30min. Then reaction was stopped by fixing the cells in 4% paraformadehyde at 4℃ for 15min, subsequently permeabilised in 0.1% Triton (in 1% BSA/PBS) for 2min on ice. After that cells were incubated in 10% donkey serum (in 1% BSA/PBS) at room temperature for 1h and then labelled by 1:50 anti-P-EGFR (tyr1173) at 4℃ overnight. The next day, cells were labelled by 1:200 donkey anti-rabbit cy5 at room temperature for 1h in the dark and the nucleus was labelled by incubation of the cells with 5mM SYTOX for 30s at room temperature. Finally the coverslip was mounted on a slide and images were captured by confocal microscope. Images are representative of 3 independent experiments.

6.2.3.3 Recovery of the internalised EGFR activity and its effect on the phosphorylation of Akt and ERK

The treatment of AG1478 blocked EGF stimulation of Akt and ERK phosphorylation and hepatocyte cell cycle progression (Chapter 3). Furthermore, AG1478 did not inhibit ligand-mediated EGF/EGFR complex internalisation (Figure 6.12-6.15). One interpretation is that AG1478 caused the accumulation of non-activated EGF/EGFR complexes inside of hepatocytes. Therefore, to further study the effect of internalised EGF/EGFR complexes, cells were exposed to EGF with AG1478 to allow the internalisation of non-activated ligand/receptor complexes, followed by removing both AG1478 and surface bound EGF. Cells were then further incubated for several minutes to allow the recovery of the activation of internalised EGF/EGFR. At this position, any detected stimulation response is due to the internalised EGF/EGFR complex.

In the experiment, cells were pre-incubated with AG1478 for 15min before being exposed to EGF (3nM) for 25min to allow the process of the internalisation of EGF and EGFR. Subsequently, AG1478 and EGF were both removed by 3 washes with SF medium (with or without AG1478, as indicated) with a 2min gap between the second and third washes to give time for AG1478 inside the cells to come out. Following these washes the cells were incubated for another 5min with no further additions. The 5min incubation was chosen, since this time it gives a strong stimulation of Akt and ERK phosphorylation.

Figure 6.16 shows that after 3 washes the cells were still able to respond to EGF (Line 3). When EGF/EGFR complexes had internalised in the presence of AG1478, and then both extracellular EGF and AG1478 were removed, signalling occurred from the internalised EGF/EGFR complexes. This is endosomal signalling and can be seen with response to the ERK pathway. The removal of AG1478 from the cells did not result in the complete recovery of the EGF stimulated phosphorylation of ERK, which was returned to 53.3% ± 13.8% (Figure 6.16B, Line 5). There is no restoration of Akt phosphorylation detected in the experiment (Figure 6.16A, Line 5). When the cells were washed with medium containing AG1478, there was neither Akt nor ERK phosphorylation detected (Line 6).

If the signalling, which was detected after removal of AG1478, was due to the activation of endosomal EGF/EGFR complexes, then this internalised EGFR should at this point become phosphorylated. Figure 6.17A shows the time course of the effect of the re-activated internalised EGF/EGFR complex on EGFR, Akt and ERK phosphorylation in hepatocytes. In order to compare with, the EGF stimulation of EGFR, Akt and ERK phosphorylation time course was also measured in the same experiment, as shown in Figure 6.17B. It shows that after removing the AG1478, the phosphorylation of EGFR and ERK was recovered, while Akt phosphorylation response was not detected. Moreover, the duration of the internalised EGF/EGFR stimulation was shorter than normal EGF stimulation. The internalised EGF/EGFR induced the phosphorylation of EGFR and ERK peaked at 5min and then gradually decreased in the following 1h; while normally EGF stimulated EGFR and ERK phosphorylation continually increased in the first hour.

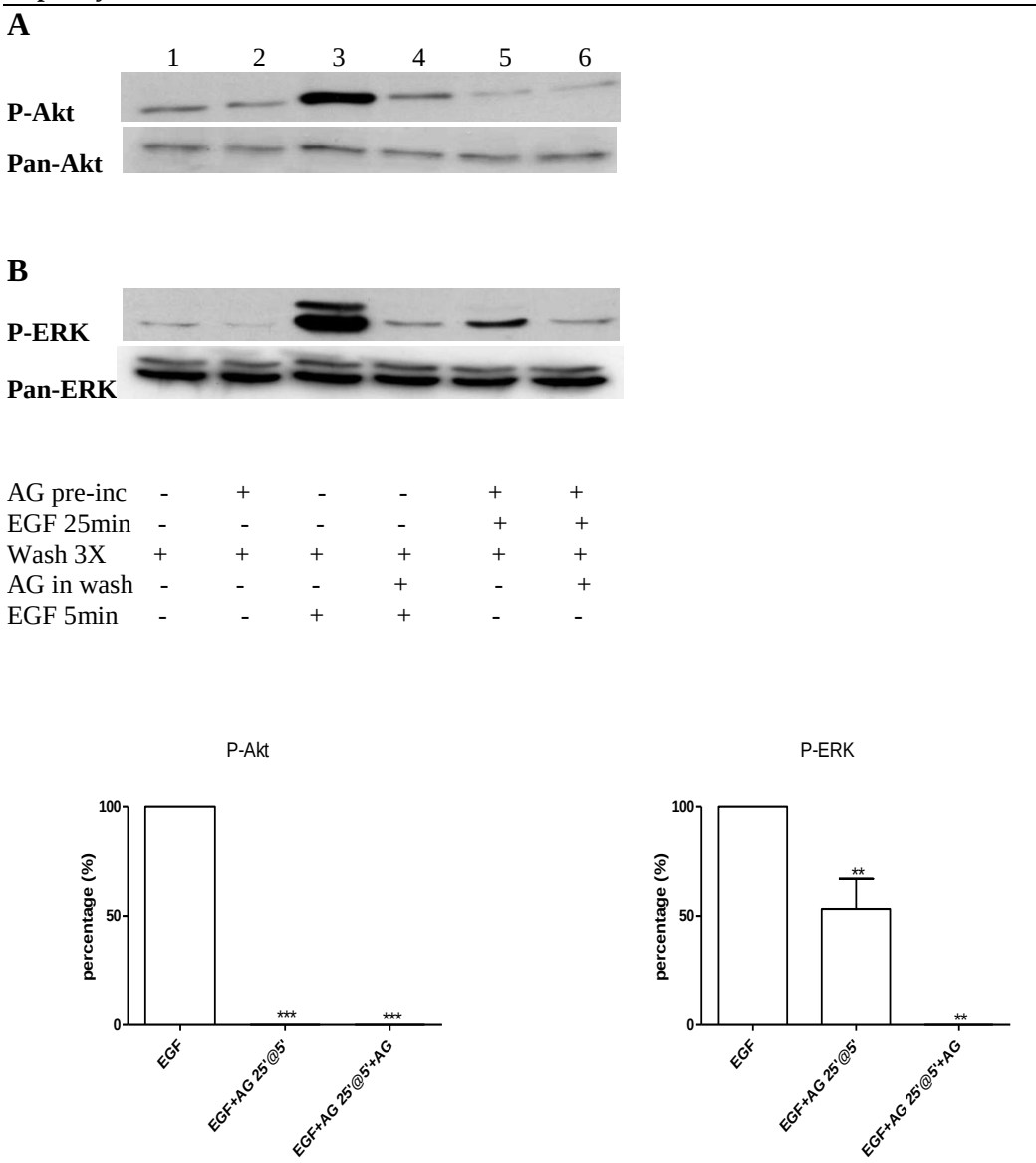


Figure 6.16 Recovery of the internalised EGF stimulation of A. phospho-Akt and B. phospho-ERK by removal of AG1478

Primary rat hepatocytes were cultured in WME supplemented with ITS and 10% FCS after plating for 4h and then incubated in SF WME for the following 48h. Cells were then pre-incubated with 300nM AG1478 for 15min and then exposed to 3nMEGF in well number 5 and 6 for 25min. After that well 5 and 6 were washed twice with SF medium, which with and without AG, respectively, and left 2min before the third wash. Cells were then left in the third wash and further incubated for 5min, in the mean time 3nM EGF was added in well number 3 and 4 and stimulated for 5min. Western blotting of phospho-ERK, phospho-Akt, pan-ERK and pan-Akt were measured. Data represented mean ± SEM (n=3) and were analysed by One-way ANOVA and post test was measured with Dunnett to compare all columns vs control column using Graphpad Prism (**p<0.01, ***p<0.001). Western blots are representative of 3 independent experiments and statistic data are from 3 independent experiments.

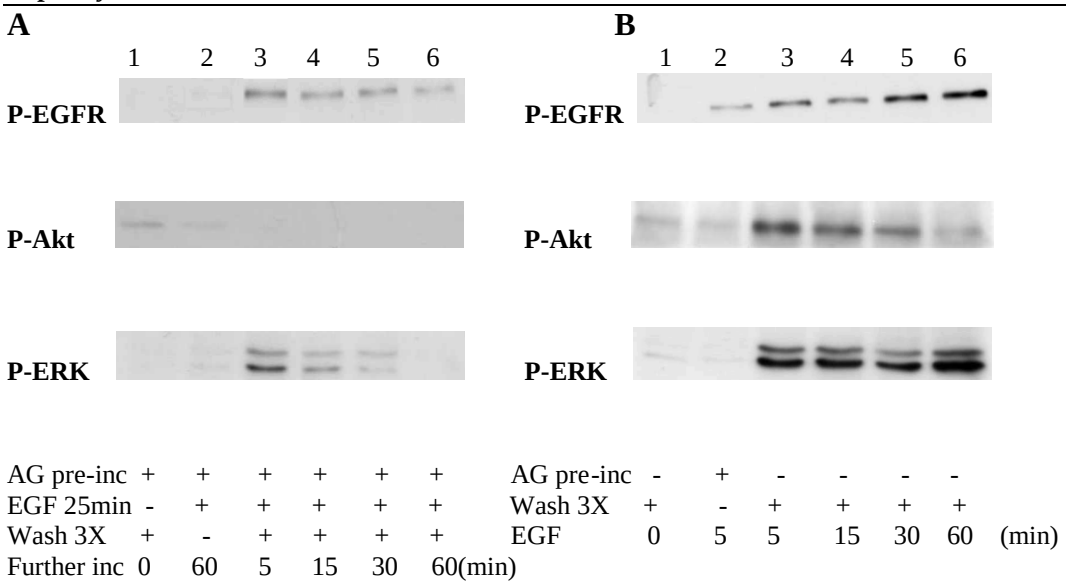


Figure 6.17 Time course of A. internalised EGF/EGFR and B. EGF stimulation of EGFR, Akt and ERK phosphorylation

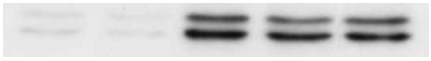
Primary rat hepatocytes were cultured in WME supplemented with ITS and 10% FCS after plating for 4h., followed with incubation in SF WME for the next 48h. Cells were then pre-incubated with 300nM AG1478 for 15min and then exposed to 3nM EGF (A) as indicated. After that cells were washed twice with S.F medium and left 2min before the third wash as labelled. Cells were then left in the third wash (A) or exposed to 3nM EGF (B) for different time as labelled. Western blotting of phospho-ERK, phospho-Akt and phospho-EGFR were measured. Western blots are representative of 2 independent experiments.

6.2.3.4 Effect of the method used to remove AG1478

The previous results (Figure 6.16 and Figure 6.17) show that the phosphorylation of Akt did not recover after removing the AG1478 while ERK phosphorylation and tyrosine phosphorylation of the EGFR were partially recovered. Since the washing system played an important role in the experiment, the effect of the washing system which used in previous (Figure 6.16 and Figure 6.17) experiment was investigated. Firstly, whether the recovery ERK phosphorylation response was due to remaining surface binding EGF was studied. In order to answer this question, an extra acid wash was provided to remove the maybe surface binding of the EGF. Secondly, whether three times wash provided in the experiment is sufficient to remove all the AG1478 from hepatocytes was investigated. In order to answer this question, cells were incubated with 300nM AG1478 for 15min, after that AG1478 was removed by the wash system, followed by exposure of the cells to EGF for 5min. This EGF stimulation was then compared to the EGF-induced response in control cells, which were treated with three washes in the same way but without AG1478.

In Figure 6.18, cells were exposed to ice-cold 0.5M NaCl/0.2M acetic acid for 5min before three washes with S.F medium to remove the surface-binding EGF. It shows that after the acid wash cells were still able to response the EGF stimulation of ERK phosphorylation (Line 3). Furthermore, internalised EGF/EGFR activation was recovered after the wash and stimulated the ERK phosphorylation (Line 5). The response was similar to that seen in Figure 6.17, when cells were only washed with S.F medium but no acid wash. This suggested that the wash system (no acid wash) we used in previous experiment was able to remove the surface binding EGF in hepatocytes.

As shown in Figure 6.19, although cells still response to EGF stimulation of Akt and ERK phosphorylation after removal of AG1478 by this wash system, it almost abolished EGF-induced Akt phosphorylation (4% ± 1%) and significantly reduced the level of ERK phosphorylation to 40% ± 10%. This suggests that some AG1478 may remain after the wash and partially inhibited EGF stimulation in hepatocytes.



AG pre-inc	-	+	-	+	+
EGF 25min	-	-	-	-	+
Acid wash	+	+	+	+	+
Wash 3X	+	+	+	+	+
EGF 5min	-	-	+	+	-

Figure 6.18 Effect of acid wash on EGF stimulation of ERK phosphorylation

Primary rat hepatocytes were cultured in WME supplemented with ITS and 10% FCS after plating for 4h., followed with incubation in SF WME for the next 48h. Cells were then pre-incubated with 300nM AG1478 for 15min and exposed to 3nM EGF for 25min as indicated. Then, cells were washed once with cold S.F medium, cold 0.5M NaCl/0.2M acetic acid (5min on ice) and another time of cold S.F medium. Subsequently, cells were washed three times with S.F medium with a 2min gap between the second and third wash Cells were then further incubated with/without re-exposed to 3nM EGF for 5min as labelled. Western blotting of phospho-ERK was measured. Western blots are representative of 2 independent experiments.

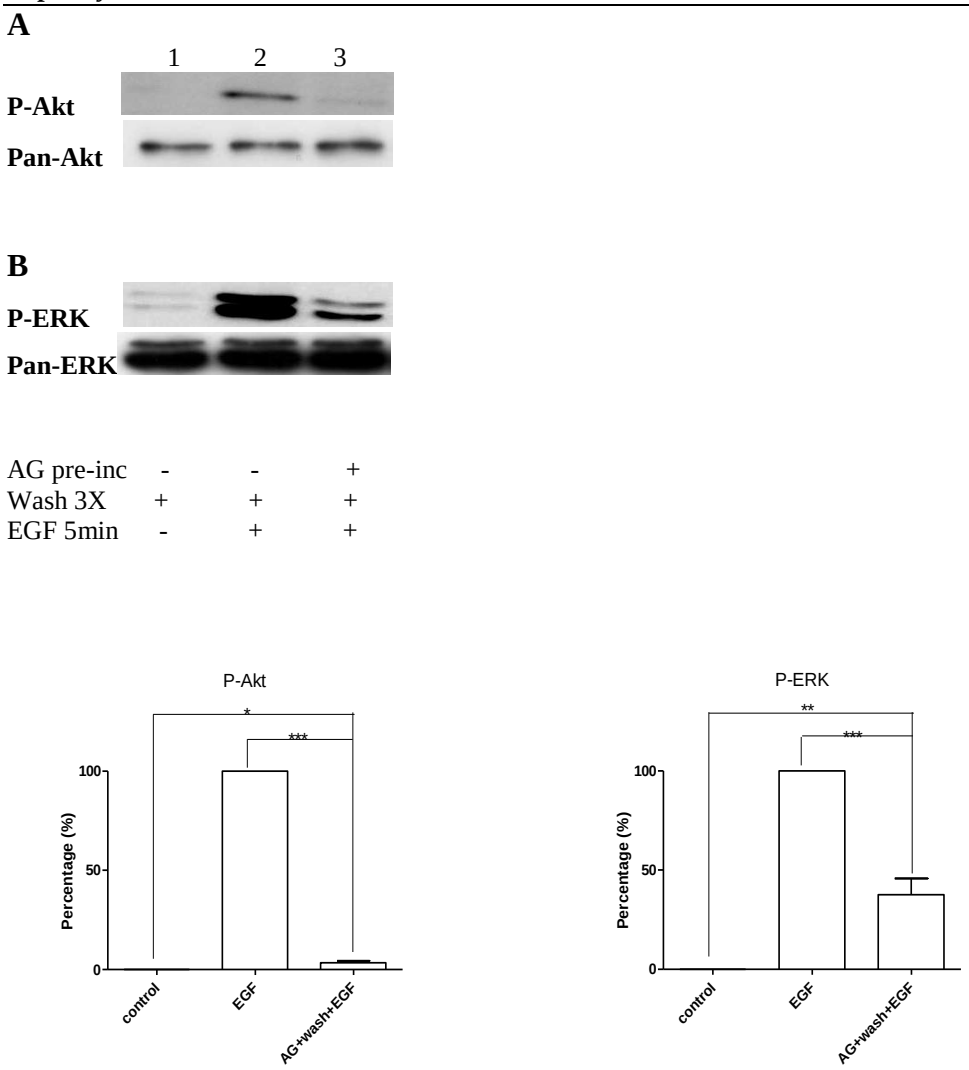


Figure 6.19 Effect of the EGF on A. phospho-Akt and B. phospho-ERK after removal of AG1478 by the wash system

Primary rat hepatocytes were cultured in WME supplemented with ITS and 10% FCS after plating for 4h., followed with incubation in SF WME for the next 48h. Cells were then pre-incubated with 300nM AG1478 for 15min in well number 3. Cells were then washed three times with S.F medium with a 2min gap between the second and third wash. Cells were then exposed to 3nM EGF for 5min at well number 2 and 3 as indicated. Western blotting of phospho-ERK, phospho-Akt, pan-ERK and pan-Akt were measured. Data represented mean ± SEM (n=3) and were analysed by One-way ANOVA and post test was measured with Bonferroni's to compare all data sets using Graphpad Prism (*$p<0.05$, **$p<0.01$, ***$p<0.001$). Western blots are representative of 3 independent experiments and statistic data are from 3 independent experiments.

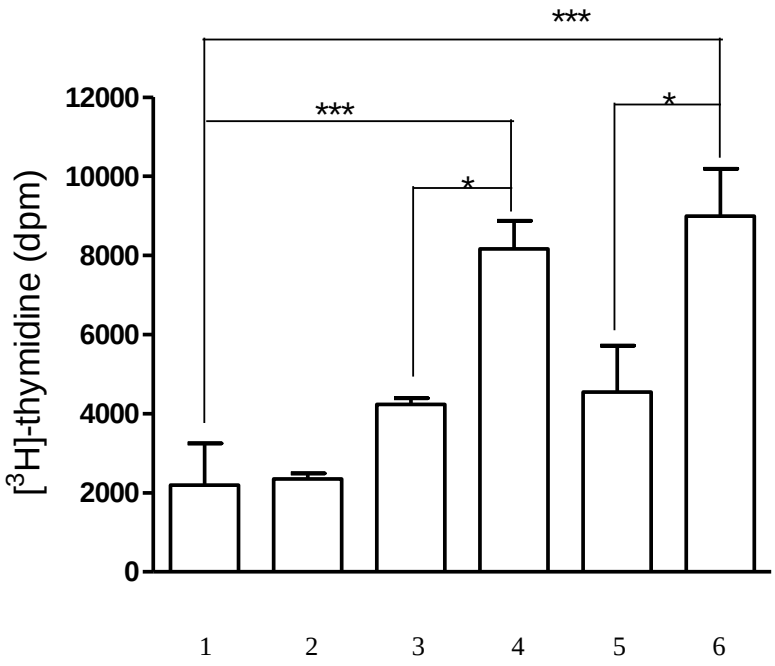
6.2.3.5 Recovery of the internalised EGFR activity and its effect on the primary rat hepatocytes cell cycle progression

In order to investigate whether the internalised EGF/EGFR can induce the cell cycle progression, cells were exposed to EGF with AG1478 present to achieve the internalisation of unactivated EGF/EGFR complexes. Then the EGF and AG1478 were removed by the wash as described in previous experiments (Figure 6.16 and Figure 6.17) and further incubated for 24h before measuring the level of [^{3}H]-thymidine incorporation into DNA.

With this experimental design, recovery of the internalised EGF/EGFR activity by washing out AG1478 did not induce cell cycle progression in primary rat hepatocytes in our experiment (data not shown). However, Wang and his colleagues reported that endosomes-localised EGF/EGFR activation only lasted for less than 2h in MDCK and BT20 cells (Pennock and Wang, 2003). Therefore, the failure of the internalised EGF/EGFR stimulation of hepatocyte entering S phase may be due to the short stimulation. Fortunately, it has been shown that the continuous exposure of the cells to growth factors can be replaced with two short pulses of growth factors (Jones and Kazlauskas, 2001). They claimed that the first stimulation moves the cell through the G_0 to G_1 phase at which cells can be responsive to the second pulse of growth factor. The second pulse of growth factor is provided to the cells during late G_1 and then moves cell into S phase. In many cells types, including hepatocytes, the G_1 phase lasts approximate 7-9h. Therefore, in the experiment, the cells were exposed to EGF for 1h, followed by incubation in agonist-free SF medium for 7h, then the second exposure of cells to EGF for 16h. During the last 4h of this total 24h (1h+7h+16h) incubation, cells

were labelled with [^{3}H]-thymidine and the level of [^{3}H]-thymidine incorporation was measured.

Figure 6.20 shows that only first pulse of the EGF (Column 2) or the second pulse (Column 3) alone could not significantly induce cell cycle progression in primary rat hepatocytes. The two short pulses (1h in the beginning and 16h in the end of a total 24h incubation) of EGF (Column 4) triggered hepatocytes to enter S phase, suggesting both first and second exposure were necessary for the cell cycle progression. Column 6 shows the result of when the first stimulation was provided by internalised EGF/EGFR. In this column, cells were exposed to EGF with AG1478 present to achieve the internalised EGF/EGFR, followed by removal of both agonist and inhibitor using our washing system. Therefore, when cells were then further incubated for 1h, the stimulation was provided by the internalised EGF/EGFR complexes. The first stimulation was terminated by 3 washes of the cells with S.F medium and further incubated in the last wash for 7h. The second pulse of EGF was then provided and left for 16h before [^{3}H]-thymidine assay. It shows that the internalised EGF/EGFR complexes successfully moved quiescent hepatocytes to G_1 phase and could be responsive to the second stimulation which then triggered cells entering S phase.



AG1478 preinc	-	-	-	-	+	+
EGF 30min	-	-	-	-	-	+
Wash x 3	+	+	+	+	+	+
1st EGF pulse 1h	-	+	-	+	-	-
Wash x 3	+	+	+	+	+	+
2nd EGF pulse 16h	-	-	+	+	+	+

Figure 6.20 Effect of the internalised EGF/EGFR on hepatocyte cell cycle progression

Primary rat hepatocytes were cultured in WME supplemented with ITS and 10% FCS after plating for 4h., followed with incubation in SF WME for the next 24h. Cells were then pre-incubated with 300nM AG1478 for 15min in Column 5 and 6 and then added 3nM EGF to Column 6 and left for 30min. Cells were then washed three times with S.F medium with a 2min gap between the second and third wash. After that cells were further incubated third wash for 1h with (Column 2 and 4) or without (Column 1, 3, 5 and 6) 3nM EGF present for 1h as labelled. The first stimulation was terminated by washing cells with S.F medium for 3 times and cells were further incubated in S.F medium for 7h. Then the second pulse of 3nM EGF was provided into cells at Column 3, 4 and 6 and last 16h. DNA synthesis was measured by thymidine assay (as described in Chapter Two). Data represented mean ± SEM (n=4) from a single experiment and were analysed by One-way ANOVA and post test was measured with Bonferroni's to compare selective pairs of columns using Graphpad Prism (*$p<0.05$, ***$p<0.001$). Data are representative of 3 independent experiments.

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6.3 Discussion

Inhibition of receptor internalisation by nystatin increased the UTP stimulation of Akt and ERK phosphorylation which suggests that P2Y receptor signal from cell membrane, and caveolin-mediated pathway regulates internalisation of the receptor, which results in termination of the receptor activation (Figure 6.1). Furthermore, nystatin had little effect on EGF stimulation indicated that the ligand-induced EGFR internalisation in hepatocytes is caveolin-independent. When cells were transfected to express Dn-dynamin, which inhibited clathrin dependent internalisation, EGF stimulation of Akt and ERK phosphorylation was attenuated while there was no significant effect on UTP-induced response (Figure 6.3). These results suggested that in primary rat hepatocyte, EGFR internalises via clathrin-dependent pathway and its ability of stimulation of Akt and ERK phosphorylation at least is partially dependent on the regulation of receptor endocytosis. EGF-induced EGFR internalisation is clathrin dependent (Figure 6.5), which has also been demonstrated in other cells as well. For instance, in HeLa cells, K44A dynamin mutant expression, which used to inhibit the formation of clathrin-coated pit and in turn blocked the clathrin-dependent internalisation, significantly reduced the EGF-induced ERK and PI3K activation (Vieira *et al*., 1996). Contrasting with EGF results, UTP and P2Y receptors signal from the cell surface and receptor internalise, via caveolin-mediated pathway, leading to activation termination. However, GPCR such as β2AR, 5-hydroxytryptamine (serotonin) receptor 1A (5-$HT1_A$), m1AChR, μ and δ opioid receptors were showed to internalise after ligand-binding and continued signal from endosomes or even only stimulates downstream proteins from endocytosis (McPherson *et al*., 2001b; Luttrell and Lefkowitz, 2002). This may be due to the activation of β-arrestin, which regulates the receptor binding to clathrin-coated

pits (Ferguson, 2001; Laporte *et al*., 2002), and is necessary for the receptor inducing downstream proteins (McDonald and Lefkowitz, 2001; Luttrell and Lefkowitz, 2002). This indicated that internalised GPCR may continue activation when the internalisation is clathrin-dependent. However, when the receptor internalises via the caveolin-dependent pathway it may lead to termination of the activation.

Compared to previous results, expression of Dn-dynamin did not cause a strong inhibition as when cells were treated with con A. This indicated that the almost abolishment of EGF-induced Akt and ERK phosphorylation in con A treated hepatocytes may be due to con A-mediated EGFR proteolysis rather than its inhibition of the receptor internalisation. Another explanation may be the low proportion of transfection. In the experiment, 20μl of HEK-293 cell lysis solution contacting Dn-dynamin encoded adenovirus were provided to 3ml of $1*10^5$ hepatocytes and then left 16h for RNA transcription and another 32h for protein expression. To test the transfection proportion, in another experiment certain amount (up to 50μl) of HEK-293 cell lysis solution containing green fluorescence protein (GFP) encoded adenovirus were added to 1ml of $1*10^5$ hepatocytes and treated in the same way to produce GFP in hepatocytes. The largest amount of adenovirus (50μl lysis solution containing GFP encoded adenovirus in 1ml of cells), was only transfected about 30%-40% of the cells (data not shown). Although the concentration of Dn-dynamin and GFP encoded adenovirus were different, the result still provided a possibility that only small proportion of the cells were transfected and expressed Dn-dynamin which resulting in the only small reduction but not abolishment of EGF stimulation.

The confocal images illustrate that EGF and EGFR form a complex and internalise into the cells. The internalisation could be clearly visualised after exposure of the cells to EGF for 20min and lasts about 1h. EGFRs formed a sharp and thin loop around the cell plasma membrane in unstimulated hepatocytes. It formed a thicker and faint loop after exposure of the cells to EGF within several minutes (Figure 6.9). This suggested that EGF-induced EGFR internalisation may happen in the very early stages after ligand binding to its receptor. This corresponded to another group's work who demonstrated that EGF induced ErbB1 (EGFR) rapid internalisation into endosomes, while other members of the ErbB family (ErbB2, ErbB3 and ErbB4) maintained activation for longer periods of time on the plasma membrane (Wiley, 2003). Our results also show that after exposure of the cells to EGF for 1h, most ligand and receptor were disassociated. Furthermore, EGF still appeared inside of the cells, while the EGFR was found around the cell surface although did not form the sharp and thin loop as before the stimulation. This finding agreed with the work of Nikolsky and colleagues, who showed that in A431 cells, about 25% to 30% of the EGFR recycled to the cell surface after stimulation while the rest degraded in lysosomes (Sorkin *et al*., 1991). Also, the images of phospho-EGFR (Figure 6.10) show that EGF rapidly induced the phosphorylation of nuclear EGFR and/or translocation of the phospho-EGFR into the nucleus. Furthermore, western blots (Figure 6.11) show that the EGFRs are detected in both plasma and nucleus in hepatocytes. Also the clear increase of the level of the EGFR in nucleus after stimulation suggested that EGF induces translocation of activated-EGFR into the nucleus. Nuclear EGFR was first detected in hepatocytes at 1991 in both normal and regenerating rat liver (Marti *et al*., 1991). Nuclear expression of functional EGFR was further found in many other cells and tissues such as JEG-3

cells and placenta (Cao *et al*., 1995). Nuclear EGFR/EGF might function as a transcription factor to activate genes required for proliferation by interaction with DNA transcription co-factor signal transducers and activators of transcription 3 (STAT 3) (Lin *et al*., 2001a; Lo *et al*., 2005b). The ligand-induced RTKs such as EGFR, ErbB2 and filbroblast growth factor (FGF) receptor translocation from cell surface into nucleus has been reported in many cells as well (Maher, 1996; Lin *et al*., 2001a; Giri *et al*., 2005). Moreover, back in 1987, it has been shown that EGF appeared in hepatocyte nuclei during rat liver regeneration (Raper, 1987). However, our results show that using 60nM, 488-EGF could not be detected in the cell nucleus *in vitro*. Furthermore, as shown in Figure 6.11, the apparent molecular weight of the EGFR, which was detected in the nucleus, was smaller than cytoplasmic EGFR. Notably, many groups reported that nuclear EGFR appears to be the full-length receptor (Cao *et al*., 1995; Lin *et al*., 2001a; Lo *et al*., 2005b). It is possible, therefore, that the nuclear EGFR is full length but has less phosphates comparing to cytoplasmic EGFR in freshly isolated rat hepatocytes. These may explain the inability of hepatocytes to proliferate *in vitro*, since no EGF was appeared in nucleus and the nuclear EGFR might have less phosphates.

Wang and colleagues showed that it was the receptor dimerisation rather than receptor kinase activation regulating the internalisation of EGFR (Wang *et al*., 2005). In primary rat hepatocytes, AG1478, which inhibited EGF stimulation, did not affect EGF/EGFR internalisation. In fact, inhibition of the activation of EGFR increased the internalisation response which may be due to cause the accumulation of inactivated EGF/EGFR complexes. Wang's group also reported that after internalisation of the

inactivated-EGFR, removal of both inhibitor and inducer can provide pure endocytic signal (Wang *et al*., 2002). Furthermore, after re-activation of internalised EGFR, the receptor directly stimulated further signalling at endosomes and then translocated into lysosomes for degradation without recycling to the membrane. Internalised EGFR was sufficient to activate major signalling pathways such as ERK and Akt and to provide a physiological response. From our results, internalised EGFR only partially recovered the phospho-ERK response and did not stimulate Akt phosphorylation (Figure 6.16). Wang and colleagues also showed a similar result, in which approximately 50% of the total phosphorylation (standard stimulation) of EGFR was activated following the re-activation of endosomes-associated EGFR by removing surface EGF and AG1478 (Wang *et al*., 2002). This may be because of remaining AG1478 after washing. As shown in Figure 6.19, when the cells were treated with AG1478 and this was then removed by our washing system, EGF stimulation of Akt phosphorylation was inhibited, while ERK phosphorylation was reduced to approximately 40%, which was similar as the level that induced by internalised EGFR. Moreover, acid wash produced a similar result as no acid wash (Figure 6.18) indicated that EGF binding to cell surface EGFR is weak in primary rat hepatocytes and can be easily washed away from the receptor. This may also explain why in con A treated cells, 488-EGF did not form a green loop around the cell surface (Figure 6.8D). However, although re-activated internalised EGFR did not cause the recovery of major standard EGF stimulation signalling pathways, it was sufficient to provide a physiological response. The endocytic stimulation successfully induced hepatocyte entry into the G_1 phase and able to respond to the second stimulation which triggered cells into S phase (Figure 6.20).

Chapter 7

Discussion

7.1 Introduction

The issue of hepatocyte proliferation is important, since this may be a key to treat various liver diseases. For instance, hepatocyte transplantation can help increase the survival rate of patients who have lost the ability for liver regeneration after PH, as may occur following liver resection surgery. The availability of human hepatocytes has prevented this procedure from becoming common clinical practice. Expansion of hepatocyte population in culture may provide the hepatocytes needed. Increasing the understanding of the signalling pathways by which cell surface receptors control biological effects will help to achieve the aim of producing functional proliferated hepatocytes in culture which may be used for cell therapy.

7.2 The role of PI3K/Akt/mTOR signalling pathway in proliferation related effects

The PI3K/Akt/mTOR signalling pathway plays a vital role in the hepatocytes response to EGF stimulation leading to cell cycle progression. Inhibition of PI3K, Akt or mTOR completely blocked EGF induction of cell DNA synthesis. This finding was partially in agreement with the work of Baffet and colleagues, who showed that PI3K and mTOR activity are necessary for EGF induction of hepatocyte DNA synthesis (Coutant *et al*., 2002). The same group also suggested that Akt activation is not necessary for EGF stimulation of hepatocyte cell cycle progression. However, this conclusion might be due to the low transfection rate (30%-40%) of mutant Akt (Dn-Akt) in their experiments. They concluded that Akt and mTOR are two downstream signals of PI3K while PI3K/mTOR but not PI3K/Akt is necessary for EGF induction of hepatocyte cell

cycle progression. Our results show that Akt activation is critical for EGF stimulation since inhibition of Akt activity with either inhibitor (A443654) or expression of mutant Akt (Dn-Akt) blocked EGF induction of hepatocytes entering S phase. However, the results in this thesis also show that the PI3K/Akt signalling pathway was not sufficient for the cell cycle progression, since UTP, which also activated this signalling pathway, failed to induce hepatocytes entering S phase. This might seem in contrast to our other observations (this thesis and (Luo *et al*., 2007) that expression of myr-Akt itself was sufficient to lead to cell cycle progression. However, in this case, there was prolonged and perhaps abnormally high elevated Akt activity, which may result in an 'unphysiological' response that can not achieve in nature. This means the cell cycle progression induced by over-expression of myr-Akt may be not able to be achieved by stimulating the native receptors. Nevertheless, this myr-Akt expression study did show how important Akt is for cell cycle progression. It also provided the first ever demonstration of enhancement of hepatocyte cell cycle progression by genetic manipulation (Luo *et al*., 2007). These conclusions, however, may be questioned by the suggestion that myr-Akt reduces apoptosis, as discussed later in the next paragraph.

The PI3K/Akt/mTOR signalling pathway also regulates hepatocyte survival (i.e. reduces apoptosis), as reported in many studies in the literature on many cell types (Yao and Cooper, 1995; Yao and Cooper, 1996). Expression of myr-Akt increased of cell viability in the long term incubation (MTT result) and rose the level of [^{3}H]-thymidine incorporation into the DNA. This suggested that continually activated Akt may increase cell survival and/or stimulate cell proliferation. However, the level of EGF-induced [^{3}H]-thymidine incorporation in myr-Akt expressing cells was not greater

than in non-expressing myr-Akt hepatocytes (control), and EGF did not further increase cell viability in myr-Akt expressed cells. This indicated that continual activation of Akt might increase survival rather than induce cell proliferation. However, the inhibition of Akt activity by either A443654 or Dn-Akt did not result in reduction of cell viability. Another group also showed a similar result that inhibition of PI3K/Akt/mTOR signalling pathway activity did not affect the ability of EGF to prevent hepatocyte apoptosis (Coutant *et al*., 2002). This indicated that the activation of PI3K/Akt/mTOR signalling pathway was not necessary to prevent hepatocytes in culture from entering apoptosis. However, another group showed that inhibition of the activity of this PI3K/Akt pathway blocked EGF suppression of TGF-β mediated hepatocyte apoptosis (Roberts *et al*., 2000). The different conclusion might be because Coutant's group investigated the role of PI3K/Akt pathway in EGF prevention of rat hepatocyte natural apoptosis, while the second group (Roberts) studied the role of this signalling pathway in EGF suppression of TGF-β induced cell apoptosis. Therefore, the PI3K/Akt pathway may still have an anti-apoptosis effect, depending on the conditions.

7.3 The role of MEK/ERK signalling pathway in proliferation related effects

MEK/ERK signalling pathway plays an important, but perhaps not completely necessary, role in EGF stimulation of cell cycle progression. Inhibition of ERK phosphorylation significantly reduced EGF stimulation of hepatocyte DNA synthesis but it did not abolish the response (10μM UO126 study). However, another group showed that inhibition of MEK/ERK activation lead to an abolishment of EGF induction of DNA synthesis (Coutant *et al*., 2002). This might be due to the higher

concentration of UO126 (50μM) was used in their experiment. Also UTP stimulated ERK phosphorylation in primary hepatocytes, and yet does not stimulate cell cycle progression, which suggested that MEK/ERK signalling pathway was not sufficient for cell cycle progression. The results in this thesis show that inhibition of the MEK/ERK pathway did not lead to a significant reduction of the level of DNA synthesis in un-stimulated cells. This indicated that ERK may be important to hepatocyte proliferation rather than survival. However, it was reported that ERK activity may be critical for EGF protection of hepatocytes from TGF-β induced apoptosis (Roberts *et al*., 2000), which leave the possibility that ERK may have an anti-apoptotic effect.

7.4 Hypothesis of why P2Y receptor does not induce cell cycle progression

Compared to growth factor (EGF), UTP stimulated very similar proliferation related signalling pathways such as PI3K/Akt/mTOR and MEK/ERK signalling pathways. The different biological effects induced by EGF and UTP with respond to cell cycle progression may be due the different stimulation, location and duration of the stimulated pathways. Our results indicated that it was likely that UTP/P2Y receptor stimulation occured from the cell surface, since treatment with con A did not inhibit the stimulation of the cell; if anything it increased the size of UTP stimulation of ERK and Akt pathways. EGF, on the other hand, might signal from both cell surface and endosomes. Furthermore, UTP stimulation of ERK phosphorylation was quicker and shorter than EGF stimulation. Taken with other results in this thesis, it suggestd that the EGF-induced endocytic signal prolonged the stimulation duration and this may contribute to different effect that EGF, but not UTP, induced cell cycle progression.

EGF and UTP presented a similar stimulation time course of Akt phosphorylation. However, EGF stimulation of the phosphorylation of GSK-3, a downstream protein of Akt, was more stable than the UTP induced response. This prolonged stimulation of GSK-3 phosphorylation may be a result of signalling from endosomes, compared to the cell surface. GSK-3 controls a number of regulators of cell cycle progression (e.g. cyclin D) (Diehl *et al*., 1998) so this difference in GSK-3 phosphorylation time course might contribute to the difference in regulation of the cell cycle by UTP and EGF.

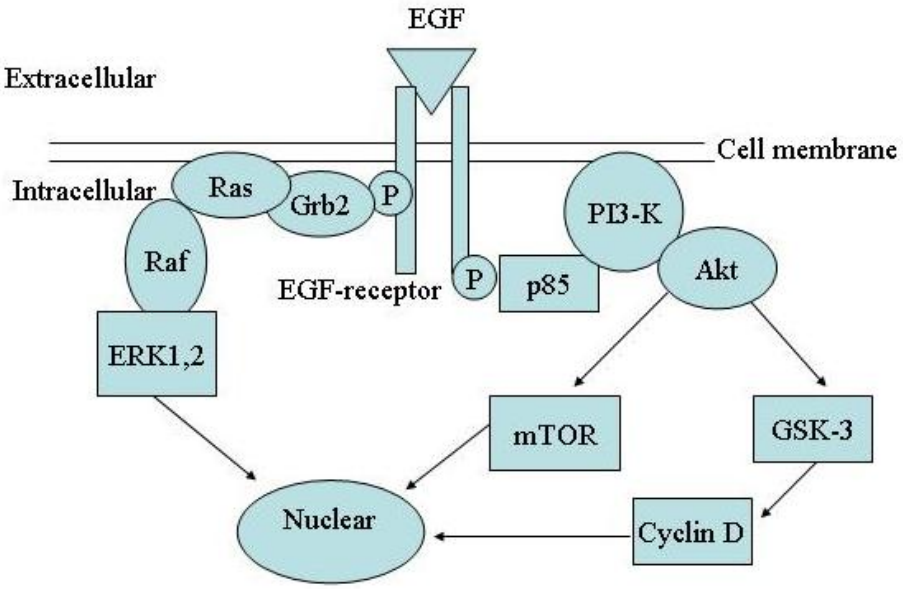
7.5 Hypothesis for the EGF stimulation of signalling pathways

Our results show that EGF binded to the EGFR on the cell surface and resulted in the homo-dimerisation of the receptor with another EGFR (ErbB1). The dimerisation of the receptor leaded to the auto-phosphorylation of the receptor at its intracellular domain and activated the receptor. Furthermore, the observation on hepatocytes in this thesis shows that the activation (i.e. phosphorylation) of the EGFR was not necessary for the EGF/EGFR internalisation. This corresponded to the conclusion of Wang and colleagues who claimed that the dimerisation but not activation of the EGFR results in the receptor internalisation (Wang *et al*., 2002). The Dn-dynamin results also show that ligand-induced EGFR internalisation was mainly via the clathrin-dependent pathway, which was in agreement with the early work of another group (Vieira *et al*., 1996). The western blotting results show that addition of EGF rapidly resulted in the phosphorylation of Akt while the induction of ERK phosphorylation was slower and more stable. According to the time course results of the EGF and EGFR internalisation (confocal work), this might be due to the EGFR inducing the phosphorylation of Akt on

or near the cell surface and that this Akt signalling terminated after internalisation. Contrasting with this, the EGF/EGFR continually stimulated ERK phosphorylation from endosomes. Other results also support the hypothesis that EGF/EGFR stimulated Akt and ERK phosphorylation from cell surface and endosomes, respectively. Inhibition of EGFR activation (with AG1478) resulted in the formation of inactivated EGF/EGFR complexes, when this was followed by removal of both the inhibitor and plasma membrane bound EGF, the signal could come only from endosomes. In these conditions, internalised EGF/EGFR induced ERK phosphorylation, but not Akt. This partially agreed with Schmid and colleagues, who reported that inhibition of EGFR internalisation resulted in a significant reduction of EGF-induced PI3K and ERK1/2 phosphorylation (Vieira *et al*., 1996). However, it remained possible that the EGF receptor may also stimulate ERK phosphorylation from the cell surface, since EGF-induced ERK phosphorylation could be detected at very early stage (1min), when a considerable proportion of the EGFRs were still on or near the cell surface. Furthermore, compared to natural EGF stimulation (i.e. no inhibitor), the level of internalised EGF/EGFR-induced ERK phosphorylation reduced to approximately 40%. However, this reduction may be also due to the remaining inhibitor. We have shown that removal of the inhibitor with the same washing system before EGF application resulted in a similar reduction of EGF induction of the ERK phosphorylation. Taking all these points into consideration, it seemed likely that EGF associated with EGFR resulted in the internalisation of EGF/EGFR complexes, and then induced ERK phosphorylation mainly from endosomes.

EGFR may also stimulate Akt phosphorylation from endosomes despite the observation that internalised EGF/EGFR alone did not resulted in the Akt phosphorylation. The failure to see Akt phosphorylation in these experiments may be due to the remaining inhibitor, after the wash being sufficient to reduce the level of EGF-induced Akt phosphorylation lower than the western blotting detectable level. We have shown that Akt was necessary for the EGF induction of hepatocyte DNA synthesis and the inhibition of Akt at the first hour of the total 24h EGF stimulation was sufficient to abolish EGF-induced cell cycle progression. Furthermore, only a small amount of activated Akt was able to achieve a complete EGF-induced DNA synthesis. The internalised EGF/EGFR stimulation at first hour plus another 16h EGF induction were sufficient to induce hepatocytes entering S phase. This indicated that internalised EGF/EGFR also stimulated a small amount of activated-Akt. As shown in the hypothesis in Figure 7.1, EGF stimulation of PI3K/Akt and MEK/ERK signalling pathways maybe from both cell surface and endosomes. Moreover, EGF simulation of Akt phosphorylation was proposed to occur mainly on the cell surface, while ERK phosphorylation was mainly at endosomes.

A The main model for Akt stimulation



B The main model for ERK stimulation

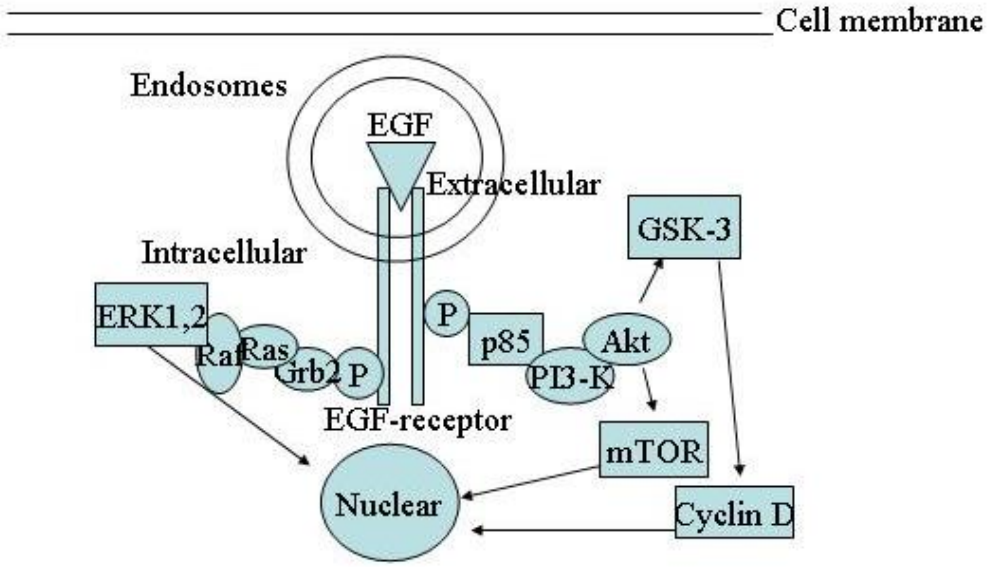


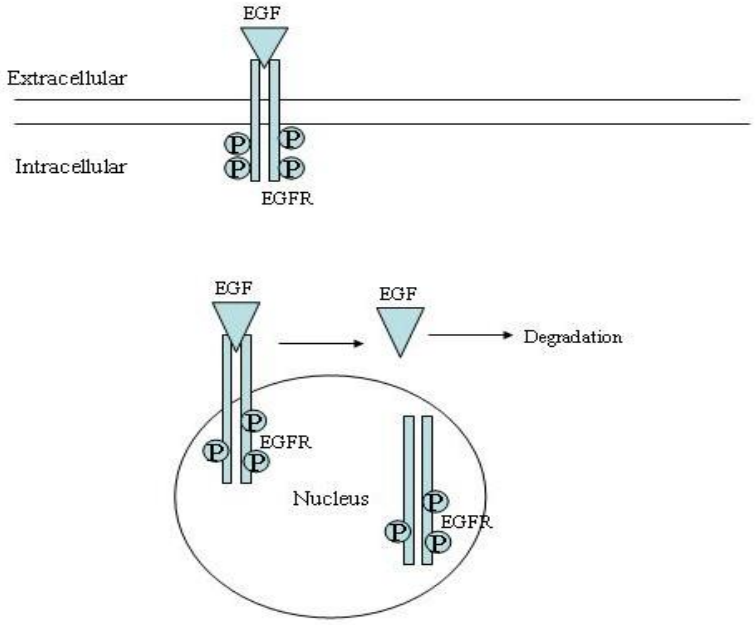
Figure 7.1 Hypothesis of EGF/EGFR stimulation signalling pathway A. from cell surface and B. from endosomes in rat hepatocytes

7.6 Hypothesis of nuclear EGFR signal

The activated EGFR was also detected in the nucleus, and these receptors may contribute to the biological effect of EGF on rat hepatocytes. Our results showed that EGFRs were located on both cell membrane and nucleus in hepatocytes and were able to be activated. Application of EGF to hepatocytes rapidly stimulated the EGFR both on the cell surface and nuclear membrane, as shown in Figure 7.2A. However, it was also possible that the phosphorylation of nuclear EGFR may be due to non-ligand induction such as stress. Following stimulation with EGF, the cell surface EGFR internalised and some translocated into the nucleus, while others recycled to the membrane or degradated (Figure 7.2B). Furthermore, EGF might be removed before the receptor translocating into the nucleus and the receptor might also lose some phosphates during the import, since 60nM 488-EGF could not be detected in the nucleus and the nuclear phosphorylated-EGFR showed a smaller apparent molecular weight on gel-electrophoresis than cytoplasmic EGFR in primary rat hepatocytes. It was possible that these issues may be related to why EGF does not stimulate hepatocyte proliferation *in vitro*. In Raper's early work *in vivo*, it was showed that EGF translocated into the nucleus during the liver regeneration (Raper, 1987). Furthermore, activated-EGFR was also detected in the nucleus of regenerating hepatocyte *in vivo* (Marti *et al*., 1991). Together these suggested that liver regeneration may involve the presence of the EGF/EGFR complexes in the nucleus. Therefore, EGF did not induce hepatocytes proliferation *in vitro* may be due to the different import mechanisms of the EGF/EGFR to nucleus between *in vivo* and *in vitro*. Further evidences also showed that in other proliferating cells or cancer cells full length EGFR and in the phosphorylation form was detected in the nucleus (Cao *et al*., 1995; Lin *et al*., 2001b;

Lo *et al*., 2005b). Therefore, these results suggested that the translocation of the full-length activated-EGFR and/or EGF into the nucleus is important for EGF full activation to induce cell proliferation. The results in this thesis, however, are the first to show the agonist-stimulated appearance of phosphorylated EGFR in cultured hepatocytes.

A



B

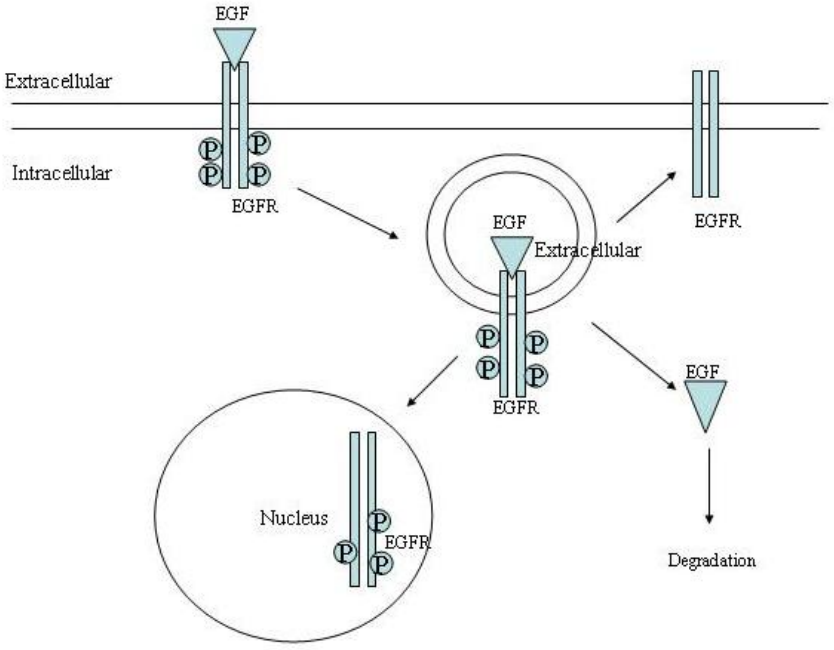


Figure 7.2 Hypothesis of A. EGF stimulation EGFR either on the cell surface or in the nucleus and B. EGFR translocation from the cell surface into the nucleus

7.7 Future work

The previous work suggested that multiple signalling pathways are involved in the regulation of hepatocyte proliferation. Increasing or maintaining activation of one signal alone, such as Akt, was not sufficiently to induce cell proliferation. Furthermore, in addition to PI3K/Akt/mTOR and MEK/ERK signalling pathway, there were other signalling pathways or signals that may play key roles in the hepatocyte proliferation. For instance, translocation of EGF and/or EGFR into the nucleus, where they may function as transcriptional activators, might be important for the cell proliferation, since EGF and EGFR was found in the nucleus of regenerating liver cells *in vivo* (Raper, 1987; Marti *et al*., 1991). It was also necessary to investigate the regulation of other signalling pathways which may be involved in hepatocyte proliferation. For example, our results show that EGF induced hepatocytes entry into S phase, but most cells are not dividing. This indicated that the hepatocyte may arrest at S phase. Furthermore, it has been shown that the apoptosis of hepatocytes following injury, such as PH, can be blocked by NO, which inhibited the activation of caspase 3 (Zeini *et al*., 2005). This suggested the possibility that some apoptotic signalling pathways may be involved and suppress hepatocytes proliferation.

The results (AG1478, AG825 study) also show that EGF stimulated EGFR homo-dimerisation with another EGFR but not EGFR ($ErbB_1$)/$ErbB_2$ hetero-dimerisation in primary rat hepatocyte. It has been shown that, compared to EGFR homo-dimer, EGFR ($ErbB_1$)/$ErbB_2$ hetero-dimerisation stimulation are more extensive and potent (Levkowitz *et al*., 1998). Furthermore, overexpression of $ErbB_2$ has been reported in many cancers such as of breast cancers (Slamon *et al*., 1987) and lung cancers (Yu *et*

al., 1994). All these suggested that the hetero-dimerisation of EGFR with $ErbB_2$ may give a more potent and extensive stimulation, which leads to cell proliferation. Therefore, the question of whether EGF stimulation $ErbB_1/ErbB_2$ hetero-dimerisation in regenerating liver (*in vivo*) should be answered. And if the answer is yes, why EGF induced this hetero-dimerisation *in vivo* but not *in vitro* could provide a good chance to achieve proliferation of hepatocytes.

The internalisation of EGFR appeared to be important for the EGF stimulation of hepatocyte proliferation. However, several questions are still remaining. First, the pathways of EGFR internalisation are still not clear. The results show that EGFR might internalise via the clathrin-dependent pathway. However, Dn-dynamin did not completely block the EGFR internalisation. This could be due to either other internalisation pathways involved, or insufficient level of virus transfection. Therefore, a reliable inhibitor or method needs to be used to further investigate the mechanism of the EGF/EGFR internalisation. Also a method of assessing level of viral transfection and degree of blocking of dynamin-dependent pathway is needed.

This thesis demonstrated the importance of PI3K/Akt/mTOR and MEK/ERK signalling pathways in EGF induction of rat hepatocyte cell cycle progression. Furthermore, many evidences have shown that signals from endosomes and translocation of the receptor into the nucleus may be crucial for EGF stimulation of cell cycle progression and/or proliferation in rat hepatocyte. The understanding of how growth factor (EGF) stimulation rat hepatocyte cell cycle progression and/or proliferation was improved.

There are also suggestions such as over-expression of activated Akt which may result in cultured hepatocytes proliferation to a level that can be used in clinic.

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